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Metabolism of ^{14}C-Histamine in Goats and Pigs Treated with Aminoguanidine

By

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Abstract

ELIASSEN K A *Metabolism of ^{14}C histamine in goats and pigs treated with amino guanidine* Acta physiol scand 1973 *88* 1—7

The effect of aminoguanidine an inhibitor of diamine oxidase (E C. 1 4 3 6) on the ca tabolism of ^{14}C-histamine has been studied in goats a species in which oxidative deamination dominates and pigs a species in which methylation is the most important route of inactivation In aminoguanidine treated goats the total ^{14}C imidazoleacetic acid (^{14}C ImAA) in urine was reduced from about 70 % to about 4 % and in pigs from 18 % to 2 % In goats the decrease in the formation of ^{14}C ImAA was partly compensated by an increased methylation though the fraction of ^{14}C histamine excreted unchanged was increased tenfold in the last mentioned species In pigs the decrease in ^{14}C ImAA seemed to be fully compensated by increased meth la tion of histamine The decrease in the excretion of non radioactive 1 4 MeImAA in pigs and the change in the ratio ^{14}C 1 4 MeImAA/^{14}C 1 4 MeHi in both pigs and goats during amino-guanidine treatment indicated that oxidation of 1 4 MeHi to 1 4 MeImAA is partly catalyzed by enzymes sensitive to aminoguanidine The incomplete inhibition of the ^{14}C ImAA forma tion during aminoguanidire treatment indicates that enzymes other than diamine oxidase might participate in the oxidation of histamine to ImAA

Histamine is oxidatively deaminated by histaminase an enzyme believed by many to be identical with diamine oxidase (E C 1 4 3 6) (Tabor 1954 Zeller 1938 and Zeller Fouts and Voegtli 1953) Histaminase is efficiently inhibited by amino guanidine (Schuler 1952 Schayer Kennedy and Smiley 1953)

Methylation is also a major pathway for histamine catabolism in many species The ensuing oxidation of the methylhistamine is mainly or entirely catalyzed by another enzyme monoamine oxidase (E C 1 4 3 4) (Rothschild and Schayer 1958) For the methylation of histamine no specific inhibitors are known

In most species *e g* rats and man the urinary excretion of histamine does not change significantly during period of aminoguanidine treatment although oxidative deamination is of great quantitative importance for the detoxication of histamine in these species (Mitchell 1956 Westling 1958 Lindell *et al* 1960 and Granerus Wet terqvist and White 1968) In sheep on the other hand the toxicity of histamine is completely altered by aminoguanidine treatment (Sjaastad 1967) This is in ac

cordance with the observations that ruminating species detoxicate iv injected histamine mainly by oxidative deamination (Eliassen 1969 and 1971 a) One of the main intentions of this paper was to examine if the observed effect of amino gaunidine in sheep is typical for the ruminating species and further to examine how histamine is catabolized when diamine oxidase is inhibited by aminoguanidine

Since there seems to be some controversy regarding the specificity of aminoguanidine on histamine metabolism experiments on this aspect are also included

It was believed that a possible effect of aminoguanidine on metabolic pathways other than oxidative deamination could be most easily demonstrated in species in which oxidative deamination is of minor quantitative importance Pigs were chosen for this purpose since methylation prior to oxidative deamination has previously been found to be the major catabolic pathway for histamine detoxication in this species (Eliassen 1971 b)

Abbreviations

Hi histamine 4(5) (β aminoethyl)imidazole AcHi N acetylhistamine 4(β acetylaminoethyl) imidazole 1 4 MeHi 1 4 methylhistamine 1 methyl 4 (β aminoethyl)imidazole ImAA imidazoleacetic acid imidazole-4(5) acetic acid 1 4 MeImAA 1 4 methylimidazoleacetic acid *1 methyl imidazole 4 acetic acid AG* aminoguanidine *DAO* diamine oxidase *(E C 1 4 3 6)*

Materials and Methods

Methods and reagents in the present study were mainly the same as those previously described (Eliassen 1969 1971 a and b)

The methods used include determination of ^{14}C-metabolites by means of isotope dilution technique Bioassay of urinary histamine activity was performed on guinea pig ileum subsequent to ion exchange chromatography Conjugated histamine was determined as the increase in urinary free histamine which occurred on hydrolysis with 10 N HCl for 1 1/2 h 1 4 MeImAA was estimated by the method of Granerus and Magnusson (1965) and Granerus (1968 a)

Materials Histamine (2 ring ^{14}C) spec act. 54 mCi/mmole was obtained from the Radiochemical Centre Amersham England

1 4 MeHi and 1 4 MeImAA were synthesized in collaboration with Maj Britt Johanson Clin Physiological Laboratory Lasarettet Lund Sweden Aminoguanidine sulfate was obtained from Eastman Organic Chemicals Rochester N Y U S A

Administration of ^{14}C Hi The purity of the injected ^{14}C Hi was checked by two-dimensional paper chromatography followed by autoradiography Only one spot was detected The ^{14}C Hi was therefore without further purification dissolved in 20 ml 0 9 % NaCl and injected into the jugular vein of the goat or into an ear vein of the pig The goats were given about 45 μCi Hi and the pigs about 60 μCi Hi One μCi corresponds to 2 06 μg Hi base

Administration of aminoguanidine 1 mg per kg of aminoguanidine sulfate (calculated as aminoguanidine base) was injected i m 3 times daily The first dose was given 2 to 3 days before the injection of ^{14}C Hi and the treatment continued throughout the experimental period About 1 week lapsed between the control experiments and the start of the aminoguanidine treatment.

Animals and feeding 2 healthy female goats of Norwegian breed weighing 40 kg and 2 healthy castrated male pigs of the Norwegian "Land svin breed were used in the experiments Their body weight increased from 70 to 90 kg during the experimental period The animals were housed in metabolism cages which permitted separate collection of urine and feces Sufficient HCl to bring the pH of the urine below 2 was added to the collection bottles The goats were fed pelleted concentrates and hay The pigs diet consisted of concentrates containing the antibiotic zincbacitracin Water was freely available throughout the experimental period

Results

Urinary excretion of Hi and 1 4 MeImAA

Histamine Free and conjugated Hi was determined in urine collected the first 24 h after injection of ^{14}C Hi The results are shown in Table II the values given in terms of the base are corrected for analytical losses and for the fraction of the injected histamine that can be calculated to be excreted unchanged in the urine (Table I) At least one recovery experiment was done for each urine specimen In the pig the recovery of histamine diphosphate (10—100 μg/25 ml) was 84 3 ± 10 3 % (S D 8 expts) For N acetylhistamine (10—100 μg/25 ml) believed to be

TABLE I Quantitative analyses for histamine metabolites in the first 24 h urine of aminoguanidine treated goats and pigs after i v injection of C-histamine

Animals	Treatment	Excreted radioactivity per 24 h in of injected dose	Histamine metabolites of total ^{14}C in the first 24 h urine							
			Hi	1 4 MeHi	Im AA Free	Im AA Ribo-side	Im AA Total	1 4 MeIm AA	Conj Hi	Sum of metabolites
Goat 4	No	86	1	0 27	—	—	77	19	~0	97
Goat 4	AG	85	11	24 0	—	—	4	61	~0	100
Goat 5	No	84	1	0 32	—	—	60	22	~0	83
Goat 5	AG	75	10	27 6	—	—	4	53	<0 5	95
Pig 4	No	94	2	10 3	4	15	19	66	<1	97
Pig 4	AG	90	1	37 5	0 3	3 2	3 5	55	<0 5	97
Pig 5	No	81	3	11 4	0 4	17	17	62	<0 5	93
Pig 5	AG	80	4	36 9	0 1	1 2	1 3	69	<1	111

TABLE II Urinary excretion of Hi and 1 4 MeImAA in goats and pigs with and without treatment with aminoguanidine

Animal	Treatment	Histamine µg/24 h Total	Free	Conj	1 4 MeImAA mg/24 h	Moles 1 4 MeImAA / Moles H	C 1 4 MeImAA / ^{14}C Hi	C-1 4 MeImAA / C 1 4 MeHi
Goat 4	No	—	—	—	3 0 }	—	—	—
Goat 4	No	—	—	—	3 7 } 2 5	—	—	—
Goat 4	No	22	—*	—	0 9 }	~30	20	70
Goat 4	AG	—	—	—	2 6	—	6	2 5
Goat 5	No	5 7	—	—	1 6	—	20	69
Goat 5	AG	—*	—	—	0 9	—	5	1 9
Pig 4	No	7 000	380	6 620	65	140	30	6 4
Pig 4	AG	17 300	1 200	16 100	42	30	60	1 5
Pig 5	No	12 500	450	12 050	100	180	20	5 4
Pig 5	AG	2 800	300	2 500	32	90	20	1 9

All values are expressed as base or acid. The values are corrected for procedural losses
* Hi could not be determined by the method used since the urine showed antihistamine like activity

identical to conjugated histamine, (Tabor and Mosettig 1949), the recoveries were about the same for both species and were as low as 47 ± 5 % (S D , 6 expts) Be cause of the presence of substances with antihistamine activity in most of the urine specimens from goats in most cases neither free nor conjugated Hi could be estimat ed in these urines

1 4 MeImAA The values for urinary 1 4 MeImAA the first 24 h after injection of ^{14}C Hi are given in Table II The recovery of 1 4 MeImAA hydrochloride added to urine (300—400 µg/25—100 ml) was nearly the same for both species and for the whole material it averaged 86 ± 20 % (S D 35 expts) The values were corrected on this basis Further the values were corrected for the fraction originating from the injected ^{14}C Hi (Table I)

Excretion of radioactivity after injection of ^{14}C Hi The radioactivity recovered in urine during periods of AG treatment was not significantly different from that recovered in the control experiments (Table I) However the excretion of known ^{14}C Hi metabolites tended to be lower during the periods of AG treatment than in the control experiments

Quantitation of ^{14}C Hi metabolites in the urine Table I gives the values of the urinary ^{14}C metabolites of i v injected ^{14}C Hi before and during periods of AG treatment The metabolic patterns in the control experiments do not deviate much from those found in previous experiments (Eliassen 1969 1971 a and b) In goats AG reduced the ^{14}C ImAA (free and riboside) in urine from 70 % to 4 % of the total ^{14}C activity The decrease in the formation of ImAA by AG treatment was partly compensated by an increase in the formation of 1 4 MeImAA and even more so by an increased formation of 1 4 MeHi In spite of this compensatory increase in the methylation pathway the fraction excreted as unchanged histamine also in creased

AG also reduced the formation of ^{14}C ImAA in pigs In this species however the decrease in ImAA seemed to be fully compensated by increased methylation of histamine (Table I) However the percentage of 1 4 MeImAA formed by oxidation of 1 4 MeHi was not increased (Table I)

AG treatment reduced the ratio ^{14}C 1 4 MeImAA/^{14}C 1 4 MeHi in both species This was most pronounced in goats (Table II) In the last mentioned species the ratio ^{14}C 1 4 MeImAA/^{14}C Hi was also reduced during periods of AG treatment this was not the case in pigs Antihistamine activity made it impossible to determine urinary free Hi in goats and accordingly the molar ratio 1 4 MeImAA/Hi could not be calculated In pigs this ratio decreased during the period of AG treatment

Discussion

The present work has demonstrated that aminoguanidine has a marked effect on histamine metabolism both in pigs and goats This effect was most pronounced in

goats in which aminoguanidine (AG) reduced urinary ^{14}C ImAA from about 70 % to 4 % of the total urinary radioactivity and increased ^{14}C 1 4 MeHi and ^{14}C Hi 100 fold and 10 fold respectively (Table I) A minor increase in urinary ^{14}C 1 4 MeImAA was also observed in this species In the pig AG markedly reduced the amount of ^{14}C ImAA excreted with the urine This decrease seemed to be fully compensated by a corresponding increase in the excretion of ^{14}C 1 4 MeHi alone without any change in the fraction excreted as unchanged ^{14}C Hi (Table I) The about 10 fold increase in the urinary excretion of unmetabolized ^{14}C Hi in goats during periods of AG treatment agrees well with findings in sheep (Sjaastad 1967) Sjaastad found that urinary free Hi increased to about 12 times the control level during periods of AG treatment In pigs AG treatment did not seem to have any significant effect on the urinary excretion of either exogenous or endogenous free Hi (Table I and II) The observation of unchanged excretion of unmetabolized Hi in pigs is in accordance with findings in man (Granerus Wetterqvist and White 1968) which metabolizes in a similar way to pigs The finding that AG in the present study affected the urinary excretion of Hi in goats but not in pigs most likely reflects the minor importance of AG sensitive enzymes in the metabolism of Hi in the pig This would also be expected from the metabolic patterns of ^{14}C Hi in the two species (Eliassen 1969 and 1971 b) AG did not completely inhibit the oxidation of i v injected ^{14}C Hi to ^{14}C ImAA either in goats or pigs This has also been found to be so in rats (Westling 1958 Westling and Wetterqvist 1962) These authors found that even doses of 20—40 mg AG sulfate/kg did not completely inhibit the formation of ImAA from Hi In man however doses of 0 1 mg/kg administered orally abolished the ^{14}C ImAA formation (Granerus 1968 b) The formation of ImAA during treatment with AG is hardly due to incomplete inhibition of histaminase It is more likely that enzymes which are little sensitive to or insensitive to AG participate in the *in vivo* formation of ImAA in some species In this connection it is worth mentioning that both benzylamine oxidase an enzyme found in blood plasma of most mammals and spermine oxidase (E C 1 5 3 3) an enzyme found in plasma of ruminants are able to catalyze the formation of ImAA from Hi (Blaschko and Hawes 1959 and Buffoni and Blaschko 1964) These enzymes are inhibited by AG (Blaschko 1962) Oxidation of ^{14}C Hi to ImAA by enzymes insensitive to AG has also been demonstrated in non ruminating species (Kobayashi 1957) The increased excretion of methylated ^{14}C Hi metabolites in both species during periods of AG treatment indicates that methylation partly takes over when the oxidation of Hi to ImAA is blocked However non radioactive 1 4 MeImAA was not materially affected by AG in goats In pigs the urinary excretion of non radioactive 1 4 MeImAA seemed to be somewhat reduced during periods of AG treatment In experiments in which large doses of Hi are given i v to goats and pigs the ratios ^{14}C 1 4 MeImAA/^{14}C Hi and ^{14}C 1 4 MeImAA/^{14}C MeHi increase (Eliassen to be published) This indicates that the decreased ratio ^{14}C 1 4 MeImAA/^{14}C 1 4 MeHi during periods of AG treatment is not due to an increase of the primary substrate Hi A more likely explanation for the reduced ratio ^{14}C 1 4 MeImAA/^{14}C 1 4 MeHi during periods of

AG treatment is that AG inhibits the oxidative deamination of 1 4 MeHi to 1 4 MeImAA This would be in agreement with the observation of Rothschild and Schayer (1958) that AG treatment increased the ^{14}C 1 4 MeHi, but not the ^{14}C 1 4 MeImAA excreted in the urine of mice Further, 12 h infusion experiments in man (Granerus *et al* 1968) also gave evidence for an impaired oxidation of 1 4 MeHi during periods of AG treatment

Oxidation of 1 4 MeHi to 1 4 MeImAA by monoamine oxidase is well established (Rothschild and Schayer 1958) The impaired oxidation of 1 4 MeHi during periods of AG treatment referred to above indicates that enzymes other than monoamine oxidase participate in the oxidative deamination of 1 4 MeHi *In vitro* studies have shown that both histamine (Lindell and Westling 1957) and benzylamine oxidase and spermine oxidase (Blaschko 1959) oxidise 1 4 MeHi

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Acta physiol scand 1973 88 8—22

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Circulatory Responses *to* Stimulation *of* Left Ventricular Receptors in the Cat

By

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Abstract

ÖBERG B and P THORÉN *Circulatory responses to stimulation of left ventricular receptors in the cat* Acta physiol scand 1973 *88* 8—22

Left ventricular receptors firing in non medullated vagal afferents were stimulated in chloralose anesthetized cats by brief aortic occlusions or by intrapericardial injections of nicotine The induced reflex effects on heart rate renal and skeletal muscle resistance vessels and muscle capacitance vessels were followed and compared with the reflex effects of arterial baroreceptor stimulation Stimulation of the ventricular receptors induced a considerable reduction in heart rate almost entirely due to vagal activation and to a reflex dilatation of renal and muscle resistance vessels and of the muscle capacitance vessels For a given reflex reduction of skeletal muscle flow resistance with the two reflex mechanisms the concomitant reflex *bradycardia and renal vasodilatation was more pronounced when the ventricular receptors were* stimulated than upon activation of the arterial baroreceptors The capacitance vessels seemed however to be engaged to the same extent from the two receptor groups The reflex muscle dilatation following ventricular receptor activation was solely due to inhibition of adrenergic vasoconstrictor fibre activity with no evidence of any cholinergic vasodilator fibre activation

The functional characteristics of a group of left ventricular receptors firing in non medullated vagal afferents were recently analysed (Öberg and Thorén 1972 a b) The receptors were found to be activated by a distension or distortion of the left ventricle either caused by a severely elevated outflow resistance or by an increased diastolic filling They were also excited by various drugs like veratrum alkaloids and nicotine

In the present study the reflex circulatory responses to stimulation of the mentioned ventricular receptors have been explored These reflex effects have been compared with those caused by activation of the arterial baroreceptors In this way an estimate of the extent of reflex engagement of various cardiovascular target organs in the two reflex mechanisms could be obtained

It was considered to be of particular interest to follow the reflex influence from the ventricular receptors on the following circulatory effectors 1) *The skeletal muscle resistance vessels* since it has been suggested (*e g* Bergel and Makin 1967)

that ventricular receptors may reflexly activate the cholinergic vasodilator fibres to the skeletal muscles 2) *The renal vascular bed* since electrical stimulation of non medullated afferents in the cardiac nerve has been shown to produce particularly marked reflex effects in this circuit (Öberg and White 1970) 3) *The capacitance vessels* since it has been suggested (Neil 1962) that cardiac receptors might exert an especially strong influence on this cardio-vascular compartment

Methods

A total of 32 cats anesthetized with chloralose 30—50 mg/kg b wt were used in the present study 16 of these cats served as donor animals from which the vascular beds under study could be perfused at essentially constant pressure head (see below) while the remaining 16 cats were prepared as follows A tracheal cannula was inserted and the carotid arteries the vagal sympathetic and aortic nerves were dissected free bilaterally in the neck The aortic nerves were cut where they join the superior laryngeal nerves The animals were placed on artificial respiration and ventilated with a gas mixture containing 3—4 % CO_2 in O The thorax was opened by an intercostal incision in the fifth interspace prolonged across the sternum The pericardium was opened and the edges suspended to the chest wall by means of ligatures A snare was placed around the ascending aorta to allow for graded obstructions of the outflow from the left ventricle The opening to the thorax cavity was covered with gauze soaked in saline to reduce as far as possible fluid losses from the exposed pleural surfaces The animal was usually transfused with 15—25 ml of dextrane i v to compensate at least partly for the shrinkage of the heart which follows the thoracotomy (*cf* Rushmer Finlayson and Nash 1954)

Recordings of circulatory variables Arterial blood pressure was measured from a catheter in one subclavian artery connected to a Statham P 23 AC transducer and a Grass polygraph recorder In most experiments the left ventricular pressure was similarly measured via a catheter advanced into the ventricular cavity from the left carotid artery Heart rate was monitored by means of a tachograph which was triggered by the rapid rise of the intra ventricular pressure (or alternatively the arterial blood pressure) The responses of the renal and skeletal muscle resistance vessels were followed by recordings of perfusion pressure and the blood flows in the left kidney and in the calf muscles of one hindleg To avoid any disturbances caused by variations of blood pressure the mentioned vascular beds were perfused at an essentially constant pressure head from a donor animal For this purpose the left kidney and the calf muscles of the right hindlimb were haemodynamically isolated from the rest of the animal leaving the vasomotor nerves entirely intact

The isolation of the calf muscles was performed according to the technique earlier described by Kjellmer (1964) All tissues except for the femur the popliteal artery and vein and the sciatic nerve were severed at the level of the knee joint The femur marrow cavity was carefully plugged with cotton to obstruct possible venous collaterals from the calf muscle The popliteal artery was connected via plastic tubing to one carotid artery of the donor animal The arterial inflow pressure was measu ed from a sidebranch of the tubing while close intra arterial injections could be given via a second sidebranch The popliteal vein was similarly cannulated and the venous outflow was passed through recorder unit operating an ordinate recorder on the polygraph and then returned to the donor animal via the external jugular vein

The left kidney was isolated by caut ously f eeing the renal artery and vein from the surrounding tissue great ca e being taken not to damage the renal vasoconstrictor fibres The renal artery and vein were subsequently connected to the donor animal as described above — Resistance vessel response ere followed in terms of alterations in flow resistance expressed as $\frac{\text{mm Hg}}{\text{ml flow/min}}$

In 4 expts the response of the apac tance essels in the calf muscles were followed in terms of phasic changes of calf olume recorded with a plethysmograph c technique (Kjellmer 1964) Volume changes were mon tored via a reco d ng device on the G ass recorder In 2 expts where the calf muscle blood flow was followed the hypothalamic defence area was stimulated A unipolar steel elect ode nsulated e cept at its tip was stereotaxically introdu ed into the hypothalamic defence area and stimuli (60/s 4—6 V 2—3 ms) were delivered f om a Grass model S4 Stimulator

Mode of receptor st mulat on The left ventricular receptors were act ated either by brief partial oc lu ions of the ascending aorta se ere nough to caus an in ease of left entricular

diastolic pressure or by intrapericardial injections of nicotine (50 μg dissolved in 2—3 ml saline) subsequently eliminated by profuse saline rinsing To minimize possible compensatory reflex adjustments from the arterial baro and chemoreceptor areas during these manœuvres and particularly when the pressure fell drastically distal to the site of the aortic occlusion the aortic n rves were cut (see above) and both carotid arteries were in most cases clamped The carotid baroreceptors were stimulated simply by pulling the carotid arteries By varying the force of the pulling and by tugging one or both carotid arteries the strength of baroreceptor activation could be graded so that a fairly wide range of reflex circulatory responses were obtained

Experimental procedures Heart rate renal blood flow skeletal muscle blood flow and volume were followed continuously while the cardiac receptors and the carotid baroreceptors were alternatively stimulated This allowed for a comparison of the relative magnitude of engagement of these various circulatory effectors in the two types or reflex mechanisms After repeated tests *the adrenergic innervation of mu cle vascular bed was blocked by i a injection of guanethidine* (Ismelin® CIBA) 0 2 mg and pentholamine (Regitin® CIBA) 0 1 mg (corresponding to approximately 4 and 2 mg/kg tissue weight respectively) Receptor stimulations were subsequently repeated again In two animals the hypothalamic defence area was then stimulated before and after atropine (0 1—0 2 mg i a to the calf) to demonstrate the effects of activation of the cholinergic vasodilator fibres to the muscle —In 4 expts atropine was given i v to the recipient animal (0 5 mg/kg b wt) to block the vagal cholinergic fibres to the heart In this way the relative importance of the vagal and sympathetic accelerance fibres respectively for the heart rate responses to cardiac receptor stimulation could be elucidated

Results

Stimulation of the left ventricular receptors either by means of a partial aortic occlusion or by intrapericardial nicotine injections regularly produced a considerable slowing of the heart and a vasodilatation in both skeletal muscle and kidney Elimination of the vasodilator response by cold block of the cervical vagi as repeatedly demonstrated in two experiments indicates that this response is of reflex origin and mediated via vagal afferents —Below a more detailed account concerning the reflex circulatory responses to stimulation of the ventricular receptors is given

1) Heart rate responses The very pronounced bradycardia induced by ventricular receptor stimulation is illustrated in Fig 2 4 5 and 6 This reflex cardiac slowing appeared almost immediately upon *e g* aortic occlusion The maximum response which usually comprised a 50 to 60 % reduction of heart rate was rapidly obtained and was well maintained throughout the stimulation period In contrast stimulation *of the arterial barorereptors often caused an initial marked slowing of* the heart lasting only a few seconds after which the heart rate increased and attained a steady state after 10—20 s When the magnitude of the reflex heart rate responses were compared in the steady state situation it was regularly *found that the* ventricular receptors produced a far more powerful bradycardia than the arterial baroreceptors Thus when identical reflex reduction of *e g* muscle flow resistance were induced from the two sets of receptors the concomitant slowing of the heart was always considerably more pronounced when the ventricular receptors were activated This phenomenon is demonstrated in Fig 1 where data from all tests in this series of experiments are compiled It is also clear from Fig 1 that stimulation of ventricular receptors produces almost maximal heart rate responses at a time when only small

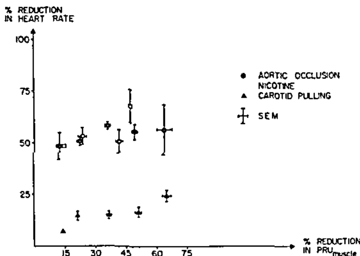


Fig 1 Diagram showing the relation between per cent decrease in muscle flow resistance and in heart rate when arterial baroreceptors and left ventricular receptors are stimulated The data are classed with regard to magnitude of resistance change in muscle Activation of the ventricular receptors by means of aortic occlusion are represented by 87 tests in eleven cats by means of nicotine administration by 13 tests in 8 cats while 63 carotid baroreceptor stimulation tests were performed in 10 cats Note the considerably greater reflex effects on heart rate for a given reflex change in muscle flow resistance when the left ventricular receptors are stimulated

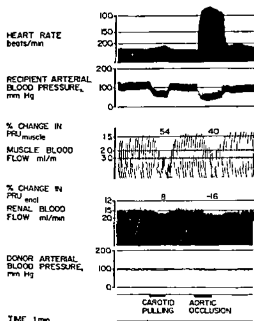


Fig 2 Reflex effects of carotid pulling and aortic occlusion on heart rate muscle and renal flow resistances The skeletal muscle and kidney were cross-perfused from a donor at constant pressure Note that the heart rate and renal vessel responses are more pronounced with aortic occlusion while the skeletal muscle response is more pronounced with carotid pulling

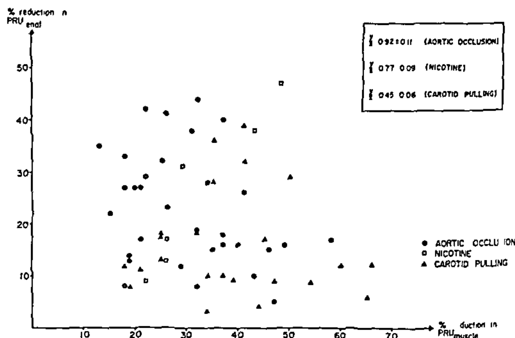


Fig 3 A Diagram showing the relationship between per cent reflex reduction in muscle flow resistance and the simultaneously obtained per cent reflex reduction of renal flow resistance upon stimulation of left ventricular receptors (by means of aortic occlusion or intrapericardial nicotine injections) and of carotid baroreceptors (by means of carotid pulling) The ratio between the renal and muscle vessel responses in each test was calculated and the mean ratio (y/x) for the three different groups of data were subsequently determined (see insert) The mean ratios for the aortic occlusion and for the nicotine data are significantly different from the ratio for the baroreceptor stimulation data

ular effects are observed This non linear correlation is an expression of the icularly strong impact of ventricular receptor afferents on central neuron pool controlling the heart rate (c f Öberg and White 1970)

The heart rate responses to aortic occlusion and nicotine administration were tested before and after atropinization of the animal (0 5 mg/kg b wt) in 4 expts *Before atropine these manoeuvres reduced the heart rate by on average* 51 and 53 per cent respectively while after atropine the corresponding figures were 5 and 8 per cent respectively The slowing of the heart in response to cardiac receptor stimulation is thus mainly due to an increased vagal influence but a certain inhibition of accelerance fibre activity also seems to take place

2) Effects on the resistance vessels in skeletal muscle and kidney Fig 2 shows records from one experiment where the renal and muscle resistance vessel responses and changes in heart rate were followed during carotid baroreceptor activation (carotid pulling) and stimulation of the ventricular receptors (aortic occlusion) Both manoeuvres produced a vasodilatation in the two vascular beds and as men

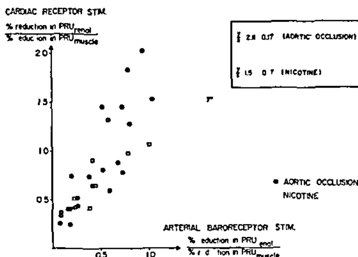


Fig 3 B Comparisons of the renal and skeletal muscle resistance vessel responses to ventricular (cardiac) receptor and baroreceptor stimulation tests performed immediately after each other in the course of the experiment ("paired stimulations") To normalize the data from all such comparisons *the effects of activation of the two receptor groups are expressed as the quotient* between renal and muscle vessel responses Each "paired" comparison is represented by one point in the diagram different symbols are used when ventricular activation was produced by aortic occlusion and by nicotine —It is seen that the data are displaced to the left and above the identity line indicating that for a given muscle vessel response in the two reflex mechanisms the renal vessel responses are more pronounced when the ventricular receptors are stimulated —The mean ratio x/y for the two groups of data presented in the diagram are statistically different from the ratio of identity (y/x = 1)

tioned a considerable reduction of heart rate The renal vascular responses are in this experiment throughout small compared with the muscle vessel responses It is clear however that the vasodilatation in the kidney is more pronounced when the ventricular receptors are activated than upon carotid baroreceptor stimulation The reverse is true for the vascular responses in the muscle This was a quite regular finding in all experiments in this series as illustrated by the compiled data in the diagram of Fig 3 A The simultaneous reflex responses of the muscle and renal resistance vessels to ventricular receptor and baroreceptor stimulation respectively are here plotted against each other In some experiments the reflex vascular adjustments to stimulation of the 2 receptor groups showed an initial peak response attained simultaneously in the 2 beds and followed by a partial recovery towards control values despite continuous receptor activations These experiments are represented by the peak response in Fig 3 A The wide scatter of the data in the diagram reflects the pronounced variations in magnitude of the response between individual animals

The data in the diagram in Fig 3 A were treated in the following way The ratio between the per cent reduction of renal and muscle flow resistance (y/x) at each individual test was calculated The mean and S E of the ratios for the aortic

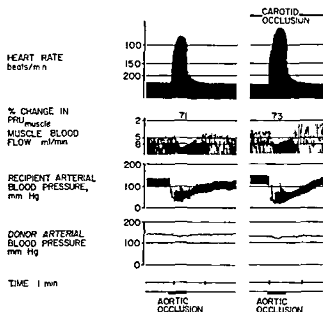


Fig 4 The reflex effects of aortic occlusion on heart rate and muscle flow resistance before and during bilateral carotid occlusion The presence of intact buffering carotid baroreceptors did not in this case of intense left ventricular receptor activation affect their reflex influence significantly

occlusion nicotine and carotid pulling data respectively were subsequently deduced The three groups of ratios were then compared in a t test which revealed that the ratio for aortic occlusion data ($x/y = 0\ 92 \pm 0\ 11$) and nicotine data ($x/y = 0\ 77 \pm 0\ 10$) *were statistically different from the ratio for the carotid pulling data* ($y/x = 0\ 45 \pm 0\ 06$) ($p < 0\ 005$ and $< 0\ 02$ respectively) These data indicate that for a given reduction in muscle flow resistance the concomitant renal vasodilatation is more pronounced when the ventricular receptors are activated than when the baroreceptors are excited —This different reflex engagement of the two vascular beds from the two set of receptors is more clearly demonstrated if comparisons are made between ventricular receptor and baroreceptor stimulation tests performed close in time in one and the same animal The results of the comparisons of such paired stimulations are illustrated in Fig 3 B The ratio between the reflex renal and muscle vessel responses to ventricular receptor stimulation is here plotted against *the same ratio obtained from the conjugate baroreceptor activation This diagram* clearly shows that the quotient between renal and muscle vessel responses is considerably higher when the ventricular receptors are stimulated *i e* the points are displaced to the left of the identity line If the data shown in Fig 3 B are statistically treated in the same way as described above (Fig 3 A) both the aortic occlusion data ($x/y = 2\ 11 \pm 0\ 17$) and the nicotine data ($x/y = 1\ 54 \pm 0\ 17$) are found to be highly significantly displaced from the identity line ($x/y = 1$) ($p < 0\ 001$ and $p < 0\ 02$ respectively)

To clarify to what extent the arterial baroreceptors were capable of buffering the reflex depressor influence from the ventricular receptors the magnitude of reflex heart rate and muscle vessel responses to ventricular receptor stimulation was

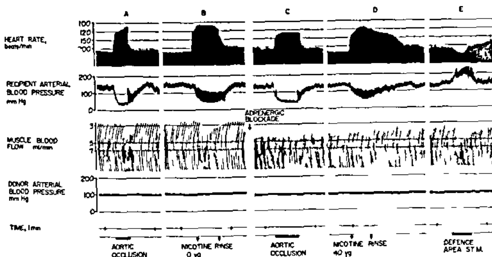


Fig 5 The effect of α adrenergic blockade on the skeletal muscle resistance vessel responses to left ventricular receptor activation The marked reflex vasodilatation in skeletal muscle upon aortic occlusion and nicotine administration (panels A and B) are completely eliminated after α adrenergic blockade (panels C and D) A marked muscle vasodilatation could still be obtained upon stimulation of the hypothalamic defence area indicating an intact cholinergic vasodilator fibre system

repeatedly compared in 6 experiments where the carotid arteries were alternatively left intact or occluded In the experiment shown in Fig 4 where a powerful activation of the ventricular receptors was produced the reflex reduction in skeletal flow resistance and in heart rate are approximately the same irrespective whether the carotid baroreceptors were allowed to display their buffering influence undisturbed or not In other experiments there was however a certain diminution of the reflex response when the carotid baroreceptor function was left undisturbed In 12 tests the mean reflex reduction in muscle flow resistance to aortic occlusion amounted to 31 per cent with intact carotid arteries and to 49 per cent when the arteries were occluded Thus, the baroreceptors are to a certain extent capable to counteract the reflex influence of the ventricular receptors except when the latter receptors are intensely activated as in Fig 4

3) *Muscle vascular responses to ventricular receptor stimulation after adrenergic blockade* In 5 experiments muscle vascular responses to ventricular receptor stimulation were analysed before and after regional α receptor blockade Records from one experiment are illustrated in Fig 5 As expected a marked bradycardia and a considerable reduction in muscle flow reisistance occur when the ventricular receptors are stimulated by partial aortic occlusion and intrapericardial nicotine administration before α receptor blockade (panels A and B) Upon regional blockade of the α receptors muscle flow resistance falls to approximately the same levels as

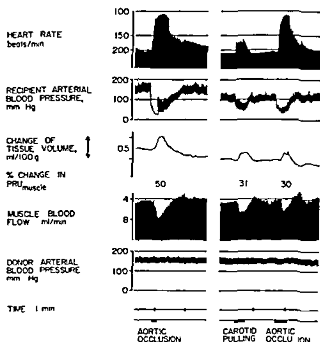


Fig 6 Effects of aortic occlusion and carotid baroreceptor stimulation on calf muscle blood flow and tissue volume Both procedures induce besides a reflex slowing of the heart a reflex dilatation of muscle resistance vessels and an augmented calf tissue volume

earlier obtained reflexly when the ventricular receptors were stimulated It thus seems as if an intense activation of these receptors is capable to inhibit almost completely the vasoconstrictor fibre tone on the skeletal muscles After α receptor blockade there is no reflex vasodilatation in the skeletal muscle in response to ventricular receptor stimulation (panels C and D) A clearcut vasodilatation ensues however upon stimulation of the hypothalamic defence area (panel E) This response was in turn abolished by atropine and was therefore in all probability due to an activation of the cholinergic vasodilator fibre system This test shows that the cholinergic vasodilator fibre system was functionally intact so that the cardiac receptors in case they made direct reflex connections with these fibres would have produced a similar atropine sensitive dilatation

Similar results as presented in Fig 5 were obtained in all five experiments in this series Thus no reflex vasodilatation of the calf muscles was ever observed after α receptor blockade despite intense activation of the ventricular receptors while stimulation of the hypothalamic defence area still produced marked dilator responses It therefore seems as if the reflex vasodilatation in the calf muscles caused by cardiac receptor stimulation is entirely due to withdrawal of prevailing sympathetic vasoconstrictor fibre activity and that there is no engagement of the cholinergic vasodilator fibres in this reflex mechanism

4) *Effects of ventricular receptor stimulation on muscle capacitance vessels* The reflex responses of the calf muscle capacitance vessels to cardiac receptor stimulation

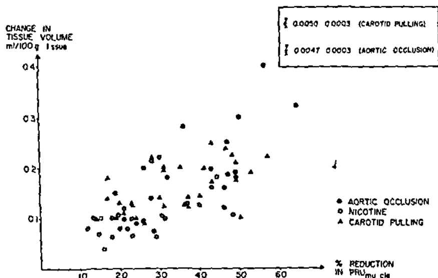


Fig 7 Relation between per cent reflex reduction in muscle flow resistance and the concomitant reflex increase of tissue volume produced by stimulations of the left ventricular receptors (aortic occlusion and nicotine) and of the carotid baroreceptors (carotid pulling) For a given reduction of muscle flow resistance the increase of tissue volume is of essentially the same order of magnitude when the two types of receptors are stimulated The mean ratios between tissue volume response and resistance response was not statistically different when aortic occlusion and carotid pulling data are compared

were studied in 4 animals and then compared with the effects produced by carotid baroreceptor stimulation Records from one expt are demonstrated in Fig 6 The response to an intense activation of the ventricular receptors caused by an almost complete aortic obstruction is demonstrated in the left panel Besides pronounced reflex reductions of heart rate and muscle flow resistance this procedure induces a fast and considerable increase of calf volume This rapid volume gain can be ascribed to a blood accumulation in the vascular bed in turn mainly due to a dilatation of the capacitance vessels (*cf* Mellander 1960) This dilatation is partly due to a reduced constrictor fibre influence on the veins but is also in part a passive phenomenon caused by the distension of the veins when the regional transmural pressure increases as a result of the relaxation of pre venous vessels In the right panel of Fig 6 a comparison is made between the reflex effects of carotid pulling and aortic occlusion This record demonstrates that when the flow resistance is reflexly decreased to approximately the same extent the dilatation of the capacitance vessels is also closely similar Data from all such comparisons are pooled in the diagram in Fig 7 where the concomitant resistance and capacitance vessel responses when the two receptor groups were stimulated are plotted against each other A statistical analysis of the same kind as utilized for the data in the diagram in Fig (see above) revealed that for a given reduction of muscle flow resistance there was no statistical difference between the magnitude of venous response when the

cular receptors and the baroreceptors were stimulated (i e no statistical difference between the mean x/y ratios) The present experiments have thus not furnished any evidence that the ventricular receptors exert a stronger reflex influence on the capacitance vessels than the carotid baroreceptors

Discussion

In a recent study (Öberg and Thoren 1972 b) a group of left ventricular receptors firing in non medullated vagal afferents were described In the present study the reflex circulatory responses to stimulation of these receptors have been analyzed Since the natural type of stimuli for the endings seems to be a *distension* of the ventricular myocardium caused by *e g* an elevated outflow resistance (Öberg and Thoren 1972 b) a shortlasting partial occlusion of the ascending aorta was in most cases used in the present experiments as a means to elicit reflex responses In some experiment however excitation of the receptors was produced by nicotine administered in the pericardial cavity

It was found that stimulation of the mentioned ventricular receptors induced a more or less generalized vasodilatation and a marked slowing of the heart due to a reflex inhibition of the sympathetic vasoconstrictor and accelerance fibre activity and an accentuation of the vagal efferent discharge to the heart These findings agree with earlier observations that elevation of the left intraventricular pressure elicits pronounced reflex inhibitory effects on the cardiovascular system (*e g* Daly and Verney 1927 Gruhzit 1955 Doutheil and Kramer 1959 Aviado and Schmidt 1959 Salisbury Cross and Rieben 1960 Ross Fraum and Braunwald *1961) These depressor responses have then usually been ascribed activation of* receptors by the raised ventricular pressure *per se* As mentioned however it seems more likely that the receptor excitation is caused by the concomitant distension of the left ventricular myocardium

It is of course possible that occlusions of the ascending aorta also lead to activation of other receptors located upstream in the left atrium in the pulmonary circulation or in the right side of the heart and that these endings then may contribute to the presently observed circulatory reflex responses Receptors in the right heart seem however to be of no importance in this connection since occlusion of the pulmonary artery produces no reflex bradycardia (*Abrahamsson and Thoren 1972) The close* resemblance between the responses obtained with aortic occlusion and with local administration of nicotine into the pericardial cavity strongly suggests that the responsible receptors are indeed located in the heart and not in the pulmonary circulation Furthermore application of local anesthetics to the ventricular epicardial surface has been found to eliminate the reflex heart rate response to aortic occlusion (Thoren 1972 b) a fact which strongly suggests that the reflex effects of aortic occlusion are initiated mainly or even solely from ventricular receptors Earlier observations also seem to support the idea that the receptors responsible for the depressor responses to aortic occlusion are indeed *located in the left ventricle*

Gruhzit (1955) thus found that selective obstruction of the outflow from the right atrium or ventricle from the pulmonary circulation or from the left atrium did not cause any significant reflex reduction of heart rate while a marked bradycardia was obtained when the ascending aorta was obstructed Essentially similar observations were made by Aviado and Schmidt 1959 Doutheil and Kramer (1959) claimed however that left atrial distension was at least equally important for inducing depressor responses upon aortic occlusion since only minor heart rate responses ensued upon this manoeuvre when the left atrial pressure was kept constant by connecting the atrium to a pressure reservoir However with this technique they probably also maintained the left ventricular enddiastolic pressure constant and hence avoided ventricular distension and consequent ventricular receptor activation when they occluded the aorta It therefore seems reasonable to assume that the presently described circulatory responses to aortic occlusion are mainly due to activation of distension receptors in the left ventricle

The reflex responses to stimulation of the ventricular receptors were in the present experiments systematically compared with those obtained when the arterial baroreceptors were activated In this way information was obtained concerning the extent of engagement of the various cardiovascular target organs such as the heart and the different vascular beds in the two reflex mechanisms Such comparisons revealed that for any given reflex reduction in muscle flow resistance the concomitant reduction in heart rate and in renal flow resistance were decidedly more pronounced when the ventricular receptors were stimulated than upon carotid baroreceptor activation In other words the afferent impulse traffic from the ventricular receptors seem to be preferentially oriented towards those central neuron pools that control the vasoconstrictor fibre discharge to the kidney and the vagal motor outflow to the heart This response pattern thus closely mimics that obtained with direct electrical stimulation of non medullated afferents in the cardiac nerve (Öberg and White 1970) One may therefore tentatively suggest that the majority of these C fibre afferents emanate from the ventricular receptors

From findings in experiments on dogs Bergel and Makin (1967) suggested that the skeletal muscle vasodilatation following activation of cardiac receptors by nicotine is at least in part due to activation of the cholinergic vasodilator fibres This finding was however not confirmed in the present study The reflex vasodilatation in the muscle was thus throughout completely abolished by α adrenergic blockade while a cholinergic vasodilatation could still be produced by stimulation of the hypothalamic defence area One reason for the apparently conflicting results besides possible species differences may be that in the study of Bergel and Makin insufficient amounts of α adrenergic blocking drugs were used so that some constrictor fibre activity which could be reflexly inhibited may still have remained This does not however explain why the reflex dilatation was very much reduced after close arterial administration of 0 6—1 mg atropine This dose appears to be quite high calculated per unit tissue weight and might have induced non specific effects

cular receptors and the baroreceptors were stimulated (i.e. no statistical difference between the mean s/v ratios). The present experiments have thus not furnished any evidence that the ventricular receptors exert a stronger reflex influence on the capacitance vessels than the carotid baroreceptors.

Discussion

In a recent study (Öberg and Thorén 1972 b) a group of left ventricular receptors firing in non-medullated vagal afferents were described. In the present study the reflex circulatory responses to stimulation of these receptors have been analyzed. Since the natural type of stimuli for the endings seems to be a *distension* of the ventricular myocardium caused by e.g. an elevated outflow resistance (Öberg and Thorén 1972 b), a shortlasting partial occlusion of the ascending aorta was in most cases used in the present experiments as a means to elicit reflex responses. In some experiments, however, excitation of the receptors was produced by nicotine administered in the pericardial cavity.

It was found that stimulation of the mentioned ventricular receptors induced a more or less generalized vasodilatation and a marked slowing of the heart due to a reflex inhibition of the sympathetic vasoconstrictor and accelerance fibre activity and an accentuation of the vagal efferent discharge to the heart. These findings agree with earlier observations that elevation of the left intraventricular pressure elicits pronounced reflex inhibitory effects on the cardiovascular system (e.g. Daly and Verney 1927, Cruhzit 1955, Douthell and Kramer 1959, Aviado and Schmidt 1959, Salisbury, Cross and Rieben 1960, Ross, Fraum and Braunwald 1961). These depressor responses have then usually been ascribed activation of receptors by the raised ventricular pressure *per se*. As mentioned, however, it seems more likely that the receptor excitation is caused by the concomitant distension of the left ventricular myocardium.

It is of course possible that occlusions of the ascending aorta also lead to activation of other receptors located "upstream" in the left atrium, in the pulmonary circulation or in the right side of the heart, and that these endings then may contribute to the presently observed circulatory reflex responses. Receptors in the right heart seem, however, to be of no importance in this connection since occlusion of the pulmonary artery produces no reflex bradycardia (Abrahamsson and Thorén 1972). The close resemblance between the responses obtained with aortic occlusion and with local administration of nicotine into the pericardial cavity strongly suggests that the responsible receptors are indeed located in the heart and not in the pulmonary circulation. Furthermore, application of local anesthetics to the ventricular epicardial surface has been found to eliminate the reflex heart rate response to aortic occlusion (Thorén 1972 b), a fact which strongly suggests that the reflex effects of aortic occlusion are initiated mainly, or even solely, from ventricular receptors. Earlier observations also seem to support the idea that the receptors responsible for the depressor responses to aortic occlusion are indeed located in the left ventricle

kidneys induced for instance reflexly by bleeding the animal or by direct stimulation of the regional sympathetic fibres thus seems to cause an increased release of renin (*cf* Bunag Page and McCubbin 1966, Coote Johns and Singer 1972) It is therefore possible that the present receptors *via* the renin angiotensin system influence the aldosterone concentrations in the blood The consequently induced changes in renal salt and water excretion in combination with the direct vasoconstrictor fibre influence on the filtration may lead to adequate compensatory salt and fluid losses via the kidney in cases of *e g* expansion of blood volume

The described reflex mechanism may also be operating in various pathophysiological states affecting the heart It is thus known that the ventricular receptors are activated by an ischemic dilatation of the heart as during a severe generalized anoxia or occlusion of one coronary artery (Thoren 1972 a) The bradycardia and hypotension sometimes observed during anoxia and coronary infarction in man may thus constitute reflex effects emanating from ventricular receptors (*cf* Thoren 1972 b c) It has also been suggested (Johnson 1971) that the bradycardia hypotensive syncope and ventricular fibrillation often seen in cases with severe aortic stenosis result from stimulation of cardiac receptors The presently described reflex mechanism may here play an important role since it is strongly activated by a severe aortic obstruction and is then capable to reduce heart rate and vessel tone and hence arterial blood pressure, to such an extent that cerebral and coronary blood flows become inadequate

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From the Department of Physiology University of Göteborg Sweden

Evidence for a Depressor Reflex Elicited from Left Ventricular Receptors during Occlusion of One Coronary Artery in the Cat

By

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Abstract

THORÉN P *Evidence for a depressor reflex elicited from left ventricular receptor during occlusion of one coronary artery in the cat* Acta physiol scand 1973 *88* 23—34

Circulatory responses to shortlasting occlusions of one coronary artery (left anterior descendent or right coronary artery) were studied in chloralose anesthetized cats — When the carotid sinus baroreceptors were left functionally intact coronary occlusion induced moderate reflex reductions of heart rate and blood pressure and slight increases of resistance and capacitance vessel tone in the calf muscles When the buffering influences from the baroreceptors were largely eliminated by bilateral carotid occlusion the reflex fall of blood pressure and heart rate became more pronounced and a clearcut vasodilatation was now observed After cold block of the cervical vagi the blood pressure fall upon coronary obstruction was markedly diminished the heart rate response was reversed to a slight tachycardia and a marked peripheral vasoconstriction ensued After i v administration of local anesthetics (lidocaine 3—4 mg/kg b wt) the reflex response to coronary obstruction and to shortlasting occlusions of the ascending aorta were almost completely eliminated — It is concluded that acute occlusions of one coronary artery induce a reflex bradycardia and vasodilatation in all probability due to activation of left ventricular mechanoreceptors The vascular responses in this inhibitory reflex seem to be significantly moderated by influences from the arterial baroreceptors

In a recent publication a group of cardiac receptors located in the left ventricle and signalling in non medullated vagal afferents were described (Öberg and Thorén 1972 a) These receptors were stimulated by a distension of the heart caused *e g* by occlusion of the aorta or by an increased venous return They were also activated after a certain latency when one main coronary artery was acutely occluded probably because of the systolic bulging of the ischemic area (Thorén 1972) Stimulation of the endings led to a reflex bradycardia and a generalized vasodilatation (Öberg and Thorén 1973)

From the mentioned observations it seemed of interest to clarify whether occlusion of one main coronary artery was capable to produce such an intense receptor activation and restraint on the vasomotor centre that clearcut circulatory depressor re

sponses were elicited. Such an analysis might have certain clinical implications since some characteristic cardiovascular responses during coronary infarction in man such as the bradycardia and the absence of a compensatory rise of flow resistance in face of a lowered blood pressure have been regarded as reflex phenomena initiated from cardiac receptors (*cf.* Zipes 1969, Weil and Shubin 1968). It was considered to be of particular interest to follow the capacitance vessel responses during coronary artery occlusion because an inadequate venous return might be one factor of importance for the emergence of the so called cardiogenic shock in man (*cf.* Weil and Shubin 1970).

It is known that the Bezold Jarisch reflex, which is probably triggered from the mentioned left ventricular receptors, can be blocked by intravenous administration of various drugs, especially local anesthetics (*cf.* Zipf 1966). Attempts were therefore made in the present study to elucidate whether the local anesthetic drug lidocaine, given i.v. in reasonable amounts, could abolish or at least diminish any reflex responses that may be induced from the ventricular receptors during coronary occlusion.

Methods

1? cats, anesthetized with chloralose 30–50 mg/kg b.wt., were used in the present study. A tracheal cannula was inserted and the carotid arteries, the vagi, the sympathetic nerves and the aortic nerves were dissected free bilaterally in the neck. The aortic nerves were cut at their junction with the superior laryngeal nerve. The vagal nerves were placed on cooling devices which could be cooled down to 0 °C by perfusion of an ice alcohol mixture. In some experiments the right vagal nerve was electrically stimulated (3–6 Hz, 2 ms, 5 V) with a bipolar silver electrode.

The abdomen was opened and the intestine removed. One cm of the abdominal aorta was dissected free distal to the renal arteries and a screw clamp was placed around it. Both splanchnic nerves were cut close to the celiac ganglion.

The animals were placed on artificial respiration and ventilated with a gas mixture containing 3–4 % CO_2 in O_2. The thorax was opened by an intercostal incision prolonged across the sternum in the fifth interspace. The pericardium was opened and the edges suspended to the chest wall by means of ligatures. A snare was placed around the ascending aorta. The left anterior descendent or the right coronary artery was dissected free for a few mm and a snare was placed around the artery to allow for shortlasting occlusion. The opening to the thoracic cavity was covered with gauze soaked in saline in order to reduce fluid losses as far as possible. The animal was given 15–30 ml of dextran after the thoracotomy in order to compensate at least partly for the shrinkage of the heart which follows the thoracotomy (*cf.* Rushmer, Finlayson and Nash 19?4).

Recordings of the circulatory variables. Arterial blood pressure was measured from a catheter in one subclavian artery connected to a Statham P 23 AC Transducer and recorded as a Grass polygraph model 5 A. Heart rate was recorded by means of a tachograph which was triggered by the rapid up-stroke of the arterial blood pressure curve. The mean arterial pressure distally to the aortic screw clamp was measured from a catheter in the left femoral artery.

Vascular adjustments were studied in the calf muscle of one hindlimb. Calf muscle blood flow was measured by cannulating the popliteal vein in the distal direction and diverting the venous outflow through a drop chamber operating an ordinate writer. In 8 experiments the reactions of the capacitance vessels in the calf were followed by means of the pletysmographic technique described in detail by Kjellmer (1964). The pletysmograph was connected to a volume recording device in turn connected to the Grass recorder.

Arterial pH was repeatedly measured (Radiometer type 27) and any tendency towards acidosis was corrected by i.v. injections of 1–3 ml 1 M $NaHCO_3$.

Experimental procedure. Changes in heart rate, blood pressure, muscle flow resistance and tissue volume were followed continuously during standardized shortlasting occlusions (30–1?0

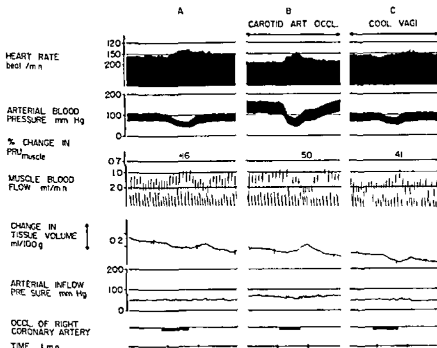


Fig 1 Changes in heart rate blood pressure muscle blood flow and tissue volume in response to occlusion of the right coronary artery when the carotid arteries were unclamped (panel A) when the carotid arteries were occluded (panel B) and during cold block of the cervical vagi (panel C) — The calf muscle perfusion pressure was maintained constant throughout

s) of one coronary artery During these experimental procedures the perfusion pressure to the hindlimb as measured from the opposite femoral artery was kept constant by suitable adjustments of the screw clamp placed around the aorta The circulatory responses to coronary artery occlusion were analyzed before and during bilateral carotid occlusion and before and after bilateral cervical vagal cooling In 6 experiments the heart rate responses to aortic occlusion coronary artery occlusion efferent vagal stimulation and pulling of the carotid arteries were tested before and after i v infusions of lidocaine Initially an amount of 3—4 mg/kg b wt lidocaine was given as a rapid infusion (3 mg/min of a 0 5 % solution) by means of an infusion apparatus followed by a lower infusion at a rate of 0 3—0 5 mg/min for 15—35 min The heart rate responses to aortic and coronary occlusions to carotid pulling and to electrical stimulation of the right vagus were again tested when this slow infusion had proceeded for some minutes The infusion was then stopped and 15—75 min thereafter the circulatory changes to aortic and coronary occlusion were again repeatedly tested

Results

In each experiment the heart rate responses to aortic occlusion were repeatedly tested Only those experiments in which a marked reflex bradycardia was obtained (more than 35 % decrease in heart rate) were subjected to further analysis Smaller effects were considered to indicate damage of the reflex pathways and such experiments were discarded In two cases markedly diminished heart rate responses to aortic occlusion were observed after placing the snares around the coronary arte

TABLE I A. Changes in blood pressure, heart rate and calf muscle resistance during short-lasting occlusions of one coronary artery with the carotid baroreceptors functionally intact. (N is number of observations.) The pre-occlusion control values for the mentioned variables are also given.

TABLE I B. Comparisons of the heart rate, blood pressure and flow resistance responses to a short-lasting occlusion of one coronary artery when the carotid baroreceptors were left functionally intact and after bilateral carotid occlusion.

TABLE I C. Comparisons of the effects of a short-lasting coronary artery occlusion performed while the vagal nerves were intact and after bilateral vagal cooling. The carotid arteries were left unclamped throughout.

	Mean arterial blood pressure			Heart rate			Calf muscle resistance		
	n	Control before coronary occl. (mm Hg ± S.E.)	Change upon coronary occl. (± S.E.)	n	Control before coronary occl. (beats ± S.E.)	Change upon coronary occl. (± S.E.)	n	Control before coronary occl. (PRU$_{100}$ ± S.E.)	Change upon coronary occl. (± S.E.)
A	45	109 2	36 [illegible]	45	[illegible]39 [illegible]	[illegible]	27	[illegible]9 ± [illegible]	-8 7
B Carotid arteries unclamped	[illegible]8	111 3	29 [illegible]	[illegible]8	[illegible]35 7	19 1	2[illegible]	26 2	+6 5
			p 0 001			p 0 005			p 0 001
Carotid arteries clamped	8	140 3	41 [illegible]	[illegible]8	[illegible]30 8	[illegible]6 [illegible]	2[illegible]	4[illegible] 3	0 5
C Intact vagal nerves	18	108 4	34 4	18	[illegible]31 10	20 3	10	[illegible]5 3	0 [illegible]
			p 0 001			p 0 001			p 0 001
Bilateral vagal cooling	18	100 3	14 3	18	[illegible]31 ± 9	-[illegible] 1	10	23	-23 8

Evidently the dissection around the vessels had injured the major part of the afferents from the ventricular receptors.

Effects of coronary artery occlusion with intact carotid baroreceptors (carotid arteries unclamped). 45 coronary artery occlusions (22 occlusions of the left anterior descendent coronary artery and 23 of the right coronary artery) lasting for 30—120 s were tested in 12 cats with unclamped carotid arteries. The typical response which appeared after a latency of 20—40 s, consisted of a decrease in heart rate and a blood pressure fall (Fig. 1, panel A). The reflex responses of the calf muscle resistance vessels were quite irregular; in some experiments a clear vasodilatation was obtained, in others a vasoconstriction. Occlusion of the left anterior descendent and the right coronary artery elicited identical response patterns. The results from all experiments in this series are therefore taken together and summarized in Table I A.

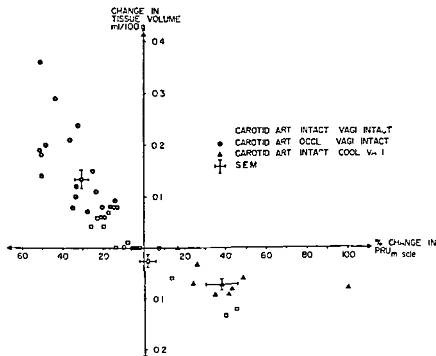


Fig 2 The changes in calf muscle flow resistance and tissue volume during occlusion of one coronary artery under different experimental conditions The results of a total of 55 occlusions in 8 cats (40 left anterior descendent and 15 right coronary artery) are shown in the diagram as well as the mean response ± S E for each of the three groups of data. The vascular responses to coronary occlusion are small when the carotid arteries were left intact (22 observations) After bilateral carotid occlusion (23 observations) a clearcut vasodilatation is observed When the cervical vagi were cooled coronary artery occlusion induced a clearcut vasoconstriction

Effects of coronary artery occlusion after bilateral carotid artery occlusion In 10 cats systematic comparisons were made between the effects of coronary artery occlusions performed before and during bilateral clamping of the carotid arteries As mentioned above and illustrated in Fig 1 occlusion of the right coronary artery elicits only moderate decreases of heart rate and blood pressure and a small vasoconstriction in the calf in case the carotid arteries were unclamped Bilateral carotid artery occlusion is seen to cause an increase of blood pressure and of calf flow resistance When the coronary artery occlusion is now repeated (panel B) there is a more marked bradycardia and blood pressure fall and a clearcut reflex vasodilatation is now observed in the calf —The results from this series are summarized in Table I B

Effect of coronary artery occlusion after bilateral vagal cooling The effect of cold block of the cervical vagi on the circulatory responses to coronary occlusion was analysed in 10 cats The carotid arteries were throughout left intact As seen in Fig

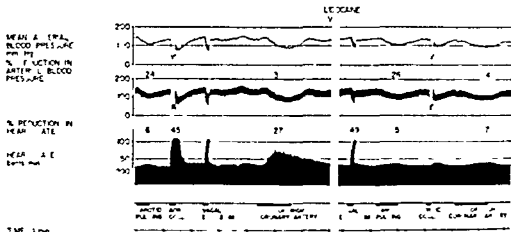


Fig 3 Changes in heart rate and blood pressure in response to occlusion of the ascending aorta efferent stimulation of the right vagus (5 Hz 3 ms CV) carotid pulling and occlusion of the right coronary artery before and after infusion of lidocaine Notice that the reflex responses to aortic occlusion and coronary artery occlusion are markedly diminished after lidocaine whereas the responses to carotid pulling and vagal efferent stimulation are essentially unchanged

1 panel C the blood pressure fall is markedly diminished and the heart rate response is reversed to a tachycardia There is also a marked reflex vasoconstriction in the calf muscles supposedly due to a decreased arterial baroreceptor activity In Table I, C the responses to coronary occlusions performed before and during bilateral vagal cooling are compared

Effect of coronary artery occlusions on the capacitance vessels The reflex responses of the calf muscle resistance vessels during coronary artery occlusion were regularly accompanied by parallel changes in capacitance vessel tone as judged from the alterations of calf tissue volume This is demonstrated in Fig 1 In 8 cats the changes in calf tissue volume and flow resistance were simultaneously followed during 50 coronary artery occlusion tests (40 occlusions of left anterior descendent coronary artery)

Fig 2 summarizes the results from the above experiments *It is seen* that as long as carotid baroreceptor mechanism is left intact the reflex changes in calf muscle resistance and regional blood content were small However after bilateral carotid occlusion a clear reflex dilatation of the resistance and the capacitance vessels ensued With cold block of the vagal nerves clearcut constrictions of the resistance and the capacitance vessels were obtained upon coronary artery occlusion

Effect of i v infusion of lidocaine on the cardiovascular changes during coronary artery occlusion In 6 cats the changes in blood pressure and heart rate to coronary artery occlusion were tested before and after i v injection of lidocaine The carotid arteries were occluded throughout to allow for enhanced reflex responses As seen

TABLE II Comparison of the effects of coronary artery occlusion on blood pressure and heart rate and of aortic occlusion on heart rate before and after i v administrations of lidocaine N S = not significant

	Coronary artery occlusion					Aortic occlusion	
	Blood pressure			Heart rate			
	N	Before occl (mm Hg±S E	Change (±S E)	Before occl (beats/min ±S E)	Change (±S E)	N	Heart rate changes (beats min ±S E)
1) Control	8	132±5	−30±3	212±18	−29±3	6	−45±3
2) Lidocaine i v	10	120±4 N S	−19±2 (p<0 001)	214±13 N S	−12±3 (p<0 005)	10	−20±4 (p<0 005)

in Fig 3 the decrease in heart rate following vagal efferent stimulation and the reflex fall in blood pressure and heart rate in response to arterial baroreceptor stimulation (carotid pulling) were not markedly influenced by the administration of lidocaine In contrast the reflex responses to coronary artery occlusion and to a partial obstruction of the ascending aorta were markedly diminished after infusion of lidocaine

In Table II data from all experiments in this series are summarized It is seen that the reflex responses emanating from stimulation of the left ventricular receptors are markedly reduced after lidocaine administration In contrast the effects of vagal efferent stimulation and of carotid pulling were not significantly different when the pre infusion control responses are compared with the effects after lidocaine infusion

15—75 min after the end of the slow infusion of lidocaine the reflex effects to aortic and coronary artery occlusion reappeared

Discussion

In the present study the reflex circulatory effects produced by a shortlasting occlusion of one main coronary artery and elicited from receptors in the heart were examined The results show that with intact carotid baroreceptors coronary artery occlusion induces moderate reflex decreases in heart rate and blood pressure while the effects on muscle resistance and capacitance vessels varied between a clear vasonconstriction and a clear vasodilatation in average muscle vessel tone increased slightly However if the carotid arteries were occluded prior to the coronary artery occlusion so that the buffering function of arterial baroreceptors was largely eliminated a more marked reflex bradycardia and blood pressure fall and clearcut reflex vasodilatation in the calf muscle were observed upon coronary artery occlusion Evidently if the buffering arterial baroreceptors are allowed to display their reflex vasoconstrictor influence undisturbed when the blood pressure falls they can effectively compete with the depressor influence from the heart receptors As a result usually only small reflex vascular reflex effects were evident upon coronary obstruc

tion The impact of the depressor reflex influence from the heart in this situation can however be clearly illustrated when the reflex pathways are blocked by vagal cooling. In this situation coronary artery occlusion elicits a marked muscle vasoconstriction probably due to a lowered baroreceptor activity when the blood pressure falls. The reflex changes in calf muscle resistance were then accompanied by proportional alterations in regional venous volume indicating that the depressor reflex influence from the left ventricular receptors considerably affects also the capacitance vessels

The present experiments also show that it is possible to block the afferent impulses from the heart by i.v. injections of lidocaine. During the rapid phase of this infusion there was in many experiments evidence for a partial blockade also of the efferent fibres to the heart but this efferent fibre blockade appeared to be abolished already a few minutes later during the slower infusion. In contrast the reflex effects to aortic and coronary artery occlusion remained reduced after i.v. lidocaine without any evidence of a blockade of the reflex responses to stimulation of the carotid baroreceptors. Thus the decreased reflex effects to aortic and coronary artery occlusion during lidocaine infusion are probably mainly due to blockade of the cardiac receptors while blockade of the vagal efferent fibres (*cf* Lieberman *et al* 1968) or drug effects on the central nervous system (*cf* Åström 1971) seem to be of less importance

Earlier studies have shown that local anesthetics can block the Bezold Jarisch reflex (Fleckenstein *et al* 1950 Hirsch *et al* 1951). For example 5 mg/kg lidocaine i.v. can abolish the effect of an i.v. injection of veratridine (Hirsch *et al* 1951). This effect is probably a direct influence upon the receptors (*cf* Zipf and Miestereck 1953 Zipf 1966). Zipf and Miestereck (1953) made the interesting observation that the continuous discharge of cardiac receptors seen in some failing hearts and also after injection of veratrum alkaloids is more sensitive to so called endoanesthesia by local anesthetics than the activity from *e g* the atrial receptors

Increased activity in cardiac receptors during experimental coronary infarction has been described by several authors (Kolatat *et al* 1967 Recordati *et al* 1971 Thorén 1972). Kolatat *et al* (1967) and Recordati *et al* (1971) recorded from medullated ventricular receptor afferents. However the more abundant receptors firing in non myelinated afferents are evidently also activated during coronary artery occusion (Thorén 1972). For several reasons (*cf* Öberg and Thorén 1972 a) these latter left ventricular mechanoreceptors seem to be by far the most important receptor group for inducing depressor reflexes from the left ventricle and are supposedly responsible for triggering the depressor responses during coronary artery occlusion as described in this study

The problem whether reflexes emanating from the heart may play a role for the circulatory adjustments in connection with experimental coronary infarction has been extensively studied. Levy and Frankel (1953) Wegria *et al* (1954) Struppler (1957), Matthes (1962) Guzman *et al* (1962) found no evidence for a depressor reflex from the left ventricle during the coronary infarction. On the contrary Agress

et al (1957 a b) described a depressor reflex with the afferent connections in the sympathetic nerves during coronary infarction in dogs but these observations have not been confirmed by others. Instead Malliani *et al* (1969) have presented evidence for a spinal reflex which increased the sympathetic outflow during occlusion of one coronary artery. Other authors again have observed a vagally mediated depressor reflex during experimental coronary infarction (*cf* Costantin 1963 Dokukin 1963 Ascanio *et al* 1965 Toubes and Brody 1970). More recently Hanley *et al* (1971) have described a vasodilatation in the skin accompanied by a vasoconstriction in the muscle during experimental coronary occlusion.

These somewhat conflicting results can probably be related to the different methods used. Coronary obstruction and/or myocardial damage has thus been induced by such different means as injection of microspheres or necrotizing substances or by occlusion of a coronary artery. The results may also vary depending on whether the thorax cavity is closed or not. Thoracotomy leads to marked shrinkage of the heart (Rushmer Finlayson and Nash 1954) which tends to unload the left ventricular receptors supposedly responsible for the reflex responses to coronary obstruction (*cf* Öberg and Thoren 1972 a). Further it may also be important whether the pericardium is intact or not since it is possible that an intact pericardium may to a certain extent prevent the ischemic bulging of the ischemic area which acts as stretch stimulus for the receptors in this situation. It is also clear from the present study that the placement of a snare or some other obstructing device around the coronary artery can easily damage the afferent fibres from the ventricular receptors since they at least partly run along these vessels.

It is of course difficult to draw any definite conclusions from the present experiments on cats as far as the situation in man is concerned. Some points of interest are however important to discuss. *Bradycardia* is a common complication to coronary infarction in man (*cf* Zipes 1969) particularly in the very early phase of the infarction. Bradyarrhythmia is thus seen in 61 % of the cases with posterior wall infarction and in 25 % with anterior wall infarction (Adgey *et al* 1968). Moreover the patients with a bradycardia response to coronary infarction often suffer from severe nausea. Since stimulation of the left ventricular receptors with non medullated afferents leads to a very pronounced reflex bradycardia (*e g* Öberg and Thoren 1973) and also to an activation of the vomiting centre (Abrahamsson and Thoren 1972 1973) it may very well be that these receptors are activated during coronary artery obstruction also in man. Since these endings are activated already within one minute after the onset of the coronary artery obstruction and since the concomitant reflex bradycardia favours the appearance of ventricular fibrillation it is possible that they contribute to the early deaths in coronary infarction in man. The reason why a bradycardia is a more common finding in posterior than in anterior wall infarctions is unknown. One possible explanation may be that the ventricular receptors are more abundant in the posterior wall in man. However in dogs and cats the endings seem to be fairly evenly distributed throughout the whole left ventricular myocardium (Sleight and Widdicombe 1965 Öberg and Thoren 1972 a)

Whether depressor reflexes emanating *e g* from left ventricular receptors are of importance for the emergence of the hypotension and the so called cardiogenic shock sometimes seen in combination with coronary infarction is no known The decrease of cardiac output due to impaired myocardial function *per se* is of course an important but not the only factor responsible for the hypotension Even if it seems to be generally agreed that systemic flow resistance is not markedly decreased during myocardial infarction there are no signs of any *compensatory increase of flow resistance* in many shocked patients (*cf* Thomas *et al* 1966 Weil and Shubin 1968 Kuhn 1970) and the combination of a decreased cardiac output and the absence of a reflexly induced compensatory rise of the peripheral resistance must necessarily lead to a pronounced blood pressure fall—This absence of an increased vasoconstrictor fibre activity may be the result of an inhibitory influence on vasomotor centre from the ventricular receptors

It has also been suggested that the low cardiac output seen in some patients with coronary infarction is primarily due to an *inadequate venous return* (*cf* Cohn 1970 Weil and Shubin 1970) A reflex venodilatation induced from cardiac receptors and leading to a more or less marked pooling in the peripheral vascular beds may here play an important role The absence of overt signs of congestive heart failure in many cases of myocardial infarction (*cf* Cohn 1970) Weil and Shubin 1970) may suggest that a reflex venodilatation and peripheral blood pooling possibly induced from the present receptors takes place in this situation

One may ask whether a blood pressure fall of the magnitude seen in cardiogenic shock is harmful or of benefit for the heart It seems likely however that a very low systemic pressure despite the reduced work load on the heart is to the disadvantage of the heart because of the further restriction of coronary flow and that therefore the hypotension should be combated One can of course not draw any direct conclusions from the present study how to treat the cardiogenic shock However some points of interest may be worthwhile to discuss If the cardiogenic shock in man is to a certain extent dependent upon a depressor reflex from the left ventricle beneficial effects should be attained by means of pharmacological interference with the effectors Thus atropine should abolish the reflex bradycardia and administration of vasoconstrictor substances should tend to compensate for the failing control of peripheral resistance and capacitance functions However a more rational approach would probably be to block the afferent limb of the postulated reflex arch. It is then interesting that according to the present results lidocaine i v can produce a rather *selective blockade of the unmyelinated left ventricular afferents* In a recent study by Ryden *et al* (1972) it was shown that 1 mg/kg lidocaine given i v during 1 min could significantly increase the heart rate for a few minutes in patients with severe bradycardia during myocardial infarction This effect can thus at least partly be ascribed to blockade of the afferent impulse traffic from activated ventricular receptors Lidocaine may of course not be the ideal or even a useful drug to induce a therapeutical blockade of the ventricular receptors during cardiogenic shock because the high doses needed to accomplish a significant blockade may

cause severe side effects It seems however to be worthwhile to search for substances with a more specific endoanesthetic effect on the ventricular receptors

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Acta physiol scand 1973 88 35—48

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Peripheral Inhibition of Eel Lateral Line Receptors as Caused by Antidromic Sensory Terminal Invasion

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Abstract

ALNÆS E *Peripheral inhibition of eel lateral line receptors as caused by antidromic sensory terminal invasion* Acta physiol scand 1973 *88* 35—48

No evidence of efferent inhibitory activity on eel lateral line sensory cells has been found in a number of different types of experiments Proximal transection of the lateral line nerve induces no changes in the ongoing activity from the sensory cells even in the uncurarized un anesthetized animal Stimulation of peripheral nerve filaments induces no inhibition of afferent spontaneous and/or evoked activity either ipsilaterally or contralaterally No spontaneous or evoked efferent activtiy could be recorded from proximal nerve filaments Antidromic invasion of sensory terminals resets the ongoing spontaneous activity In the intervening interval the mechanical and electrical thresholds of these sensory units are similarly elevated Antidromic invasion is suggested to be the phenomenon reported as peripheral inhibition by Hashimoto *et al* (1970)

The efferent synapses found on labyrinthine sensory hair cells (Wersall 1956 Engstrom and Wersall 1958 Smith and Sjostrand 1961 Wersall and Flock 1965 Hama 1969) have counterparts in vesiculated nerve endings making synaptic contact with lateral line sensory cells in a number of teleosts (Trujillo Cenoz 1961 Hama 1962 1965 Flock 1965 Pomes Delaveuve 1964 Roberts and Ryan 1971) In the labyrinthine systems these synapses have been shown to be inhibitory (Fex 1962 1967) Russell (1968) has found efferent inhibition of the spontaneously occurring activity in single *Xenopus* lateral line nerve axons and efferent action potentials have been recorded in the lateral line nerve of dogfish (Roberts and Russell 1970) Hashimoto *et al* (1970) have similarly found efferent action potentials in the lateral line nerve of the japanese sea eel (*Astroconger myriaster*) and claim that they exert an efferent inhibitory activity The present investigation was undertaken to assess the importance of possible reflex efferent activity in the eel lateral line system It is concluded however that no such activity exists and that the purported inhibition in the japanese sea eel (Hashimoto *et al* 1970) probably represents receptor depression after antidromic invasion of sensory terminals

Methods

The methods of recording and computation of activity in single sensory units in the lateral line nerve of the common eel (*Anguilla anguilla*) have been described in a previous publication (Alnæs 1972a). To achieve stable recording conditions without subjecting the sensory organs to the influence of anesthetics (MS 222) or curare, the following experimental procedure was employed. The unanesthetized fish was placed in the holder. Through a dorsal laminectomy at the level of the operculum the spinal cord was destroyed in its entire length. After removal of vertebrae the dorsal aorta was ligated at this level. Tubocurarine was injected into the proximal part of the fish in doses sufficient to permit stable recording conditions in the intracranial portion of N. lat. post. Control experiments without such proximal curarization proved the peripheral hypoxia not to have any discernible effect on spontaneous single unit activity and mechanical sensitivity of the lateral line receptors during the first 1/2 hour after ligation. By peripheral nerve stimulation in the proximally curarized fishes no muscle curarization could be detected in the distal part of the fish during the same interval.

Results

1. Absence of tonic inhibitory activity

If efferent inhibitory neurones were tonically active in the eel lateral line system one would predict an increase in spontaneous afferent firing rates to occur after central transection of the nerve. The firing rates of spontaneously active afferent units were therefore computed in preparations with and without central nervous connection, other experimental conditions being equal.

As concluded in the previous paper, two discrete groups of primary afferents exist in the lateral line nerve of *Anguilla anguilla*. The group of fast conducting fibres ($>$ 14 m/s) exhibits a different relationship between regularity of spontaneous firing (as measured by the coefficient of variation, CV) and firing frequency (as measured by mean interval $\bar{x}$) than does the group of slowly conducting afferents ($<$ 14 m/s). Fig. 1 A shows the distribution of mean intervals ($\bar{x}$) recorded in fast conducting units with intact central connections. Of 50 units investigated 38 (76%) were spontaneously active. The histogram shows a mode at $\bar{x} \sim 70$ ms and a mean $\bar{x}$ of 192 ms (SD = 64) was computed. Fig. 1 B is a corresponding histogram of units recorded after intracranial cutting of N. lat. post. Of 43 units investigated 31 (72%) were spontaneously active. The histogram shows a mode at $\bar{x} \approx 70$ ms and a mean $\bar{x}$ of 227 ms (SD = 67) was computed. No significant difference exists between the two distributions. Only 3 slowly conducting units were successfully recorded in preparations with cut central connections. (Such transection probably causes deterioration of ganglionic cells (Alnæs 1972c).) The firing frequencies of these units were *not* appreciably shifted from that of intact units. The mean $\bar{x}$ observed in the slowly conducting group was 188 ms (SD = 34).

Even though there was no change in the mean rate of spontaneous activity, the central nerve transection might conceivably change the firing pattern of the lateral line receptors. However, no differences in firing regularity (as measured by CV) was dicovered between the units recorded before and after nerve transection.

In *Xenopus* Russell (1968) has found the efferent inhibitory synapses to be blocked by curare. Stable extracellular registration in the eel N. lat. post., however, requires dependable immobilization. In 5 fishes this problem was successfully circumvented by the procedure of proximal curarization described in *Methods*.

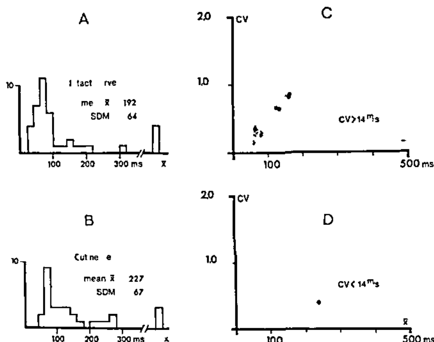


Fig 1 A Histogram of mean impulse interval in 38 spontaneously active rapidly conducting fibres B As in A for 31 similar units recorded in the same experiments after sectioning of the intracranial portion of N lat post C Scatter plot of spontaneously active rapidly conducting units Abscissa mean interval Ordinate coefficient of variation Encircled points Units in peripherally non curarized preparations D As in C for slowly conducting units

Fig 1 C and D are scatter plots of the firing characteristics of fast conducting and slowly conducting units Mean interval ($\bar{x}$) is plotted (abscissa) against irregularity of firing (CV) (ordinate) Encircled points refer to units in distally noncurarized preparations In neither calibre group are the firing characteristics altered in a measurable way by the absence of curare at the receptor site

To exclude a possible blocking effect of curare and to compare the activity of the same units with and without central connections a number of experiments were conducted also on totally unanesthetized and non curarized (but spinalized) fishes A thin filament of the peripheral lateral line nerve was dissected from the main nerve trunk and hooked on a bipolar platinum registration electrode (Fig 2) Action potentials from a large number of spontaneously active units could usually be picked up Stimulation in the proximal part of the nerve set up an antidromic nerve volley which was recorded distally in the nerve In this way it was secured that nerve damage had not taken place at the dissection locality neither fast nor slow axon components being blocked at this site The fish displayed vigorous head and neck movements and both central and peripheral circulation was normal

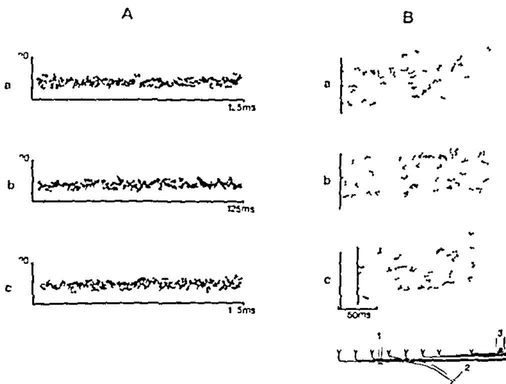


Fig 2 A Integrated activity in dissected filament of the trunk lateral line nerve Each point gives the number of action potentials to have occurred in a bin of 125/400 ms width during 200 superimposed sweeps at 1 c/s a totally unanesthetized noncurarized fish Central connection of nerve intact b 10 min after complete curarization c immediately after proximal nerve transection B Poststimulus plots of filament (electrode 1) spontaneous activity during identical time spans Each point corresponds to one action potential recorded above noise level Completely unanesthetized and noncurarized fish a spontaneous activity b single supramaximal nerve stimulation at electrode 3 occurring at vertical line C Train of stimuli delivered between vertical lines

Each action potential occurring above noise level triggered a discriminator pulse in the on line computer 200 sweeps at 1 c/s produced the histograms of integrated nerve activity shown in Fig 2 A Each dot gives the number of spike occurrences to have fallen within the respective 125/400 ms bin during the 200 sweeps that were superimposed (a) shows the amount of spontaneous activity at the start of the experiment (b) the activity 10 minutes after distal intramuscular curarization and (c) the activity registered immediately after proximal transection of the nerve

No changes in the ongoing activity could be detected in 4 similar expts nor were action potentials ever found in dissected filaments of the central stump Thus the conclusion was reached that tonic efferent inhibition plays no discernible part in the lateral line sensory cell discharge in this preparation

II Absence of evoked inhibitory activity

A number of experiments were set up to monitor possible effects of evoked efferent activity Fig 2 B shows a post stimulus plot of action potentials recorded from a peripheral nerve filament in the manner described above Each point corresponds to one action potential The entire nerve was stimulated proximally to excite possible efferents (a) shows the post stimulus plot at 0V stimulation *i e* the spontaneously occurring activity (b) shows the action potentials occurring during an identical time span after a single supramaximal proximal nerve stimulation In (c) a train of 10 such stimuli were given In no instances did a noticeable change in ongoing activity take place although a compound action potential consisting of slow and fast fibre components was successfully recorded in the distal portion of the nerve

Attempts at evoking efferent activity indirectly through reflexes were similarly unsuccessful Distal nerve stimulation did never produce efferent activity in a partially isolated proximal filament of the nerve nor did stimulation of the contralateral lateral line nerve

In all distally noncurarized fishes (as well as in the usual curarized preparations) stimulation of the contralateral lateral line nerve was never seen to modify the spontaneous or mechanically or DC induced activity of a single sensory unit Nor did ipsilateral nerve stimulation distally to the sensory field of a given unit ever produce such reflex changes (Nerve stimulation proximal to the unit would lead to antidromic invasion of its nerve fibre This phenomenon will be discussed below) The same lack of reflex efferent activity was found also for mechanical and DC stimulation of varying intensities along the lateral line canal system both in the trunk and head regions

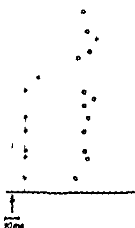
Thus the conclusion was reached that no demonstrable efferent modification of sensory cell activity takes place in this preparation either tonically or induced by reflexes This result is in contradiction with the conditions reported in the Japanese sea eel *Astroconger myriaster* (Hashimoto *et al* 1970) and *Xenopus laevis* (Russell 1968)

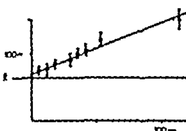
III Antidromic invasion of sensory terminals

En passage nerve stimulation as performed in the study of primary afferent influence on higher order neurones by necessity entails antidromic invasion of the peripheral terminals of the same afferents A number of experiments were performed to assess the effects of such invasion on the spontaneous and evoked impulse patterns from lateral line sensory afferents

The experiments also gave the explanation of phenomena earlier interpreted as evidence for peripheral inhibition by efferent nerve fibres in the lateral line system of the sea eel (Hashimoto *et al* 1970)

In Fig 3 A a single afferent fibre is stimulated *en passage* at its threshold in the trunk lateral line nerve Orthodromic action potentials are recorded in the intracranial portion of N lat post X in the manner described above The plot gives succeeding poststimulus sweeps from above downwards In those cases in which a





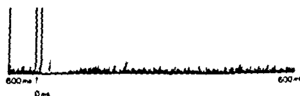


Fig 3 s From above downwards poststimulus sweeps Nerve stimulated peripherally at threshold strength spikes (dots) registered centrally Time of stimulus first vertical line (arrow) Arrival of orthodromic stimulation spike second vertical line Spontaneous spikes [ ]) wing supra threshold stimulation empty circles) Abscissa length of antidromic stimula tion train in tim interval in ms Ordinate interval from invasion of last antidromic spike to first subsequent spontaneously occurring spike Bars variation in 10 separate trial c Histogram of pre and post stimulus spike events in 100 *en passage* stimulations of spontaneous ly active afferent fibre (Abscissa Bin width ms) Time of antidromic terminal invasion rela tive to recording of spikes marked by arrow Preceding full columns refer to time of stimulus and subsequent recording of orthodromic action potential respectively Stim interval 65t ms Ordinate 100 event

stimulus has been supra threshold (orthodromic spike registered on second thin line) and an antidromic action potential has accordingly invaded the peripheral part of the axon the next spontaneously occurring action potential (circles) is seen to follow after a certain pause This pause is obviously not due to an effect of descending peripheral inhibitory axons since it occurs only when the same axon has been ex cited by the electrical stimulus The duration of the pause was related to the interval of the spontaneous activity of the unit and it was not increased by supramaximal nerve stimulation Fig 3 B shows the effect of antidromic invasion by a train of spikes in a spontaneously active fibre The length of the train of spikes in ms (6 ms pulse interval stimulus minimally above fibre threshold) is plotted on the abscissa and the ensuing pause in the spontaneous discharge is plotted on the ordinate It is seen that the first post invasion interval approaches the mean firm al of the unit ($\bar{x}$) when the train is shortened to one spike o nd increase mately l nearly with increasing spike train lengths

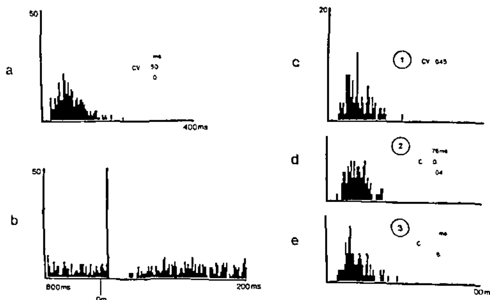


Fig 4 a time histogram of impul e intervals in spontaneously active unit 300 intervals mean interval 73 ms coeff of variation 0 50 b poststimulus spike histogram as in Fig 3 c c—e time histograms of 1st 2nd and 3rd intervals following peripheral spike invasion Legends as in a

An example of an on line computer display of post and pre stimulus events in a ingle afferent unit is given in Fig 3 C (The spike histogram is continuous both ends of the time scale are neighbouring time bins relative to stimulus) The fibre is stimulated *en passage* at 0 ms and a direct orthodromic spike is recorded at a con stant delay (bin at 10 ms) The post invasion pause and the gradual recovery of pontaneous firing in a number of sweeps are seen The arrow denotes the timing of peripheral antidromic invasion relative to the occurrence of the ensuing spontane ous spikes corrections having been made for the delay caused by the conduction of the antidromic spike to the receptor site and the lag in recording orthodromic spikes in the same fibre at the level of the medulla These relative distances and conduction velocities could be precisely determined for each single unit

By computer analysis of spontaneous discharges following antidromic invasion of the sensory terminals it was verified that such invasion causes a genuine resetting of the spontaneous recovery cycle of the spike generating mechanism In Fig 4 a and b the spontaneous interval histogram and its post invasion histogram are given With the above mentioned corrections for conduction lags in the recording proce dure statistical analysis was carried out on the interval to first spontaneous spike fol lowing invasion (Fig 4 c) and the two subsequent spike interval (Fig 4 d and e) It is seen that all intervals and their standard deviations mimic those of the spontane ous interval histogram (Fig 4 a)

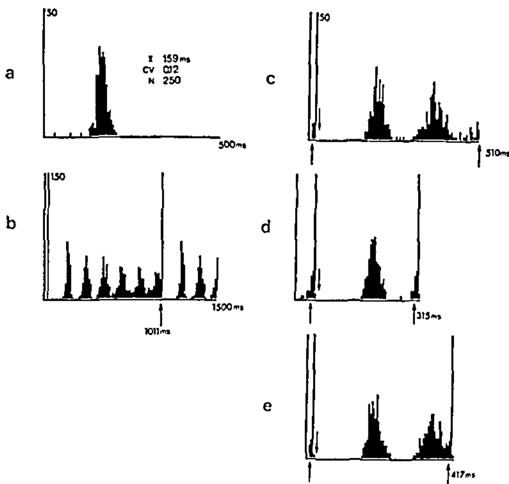


Fig 5 a time histogram of regular unit b post stimulus spike histogram Stimulus interval 1011 ms Note long lasting resetting of firing probability c—e post stimulus histograms at various stimulation intervals Descending arrow time of peripheral invasion Relative invasion timings aligned vertically Ascending arrows Time of *en passage* stimulus (stim interval in ms) Both arrows denote same relative timing

Within the statistical error margins for each individual sampling this was verified for a large number of units Resetting of spontaneous firing occurred in both thick and thin afferents and in fibres of relatively high and relatively low discharge regularities and/or frequencies

For extremely regular units it was possible to time the antidromic invasion to different probability sections of the peripheral recovery cycle An example is given in Fig 5 (a) and (b) correspond to the histograms shown for the less regular unit in Fig 4 a and b The resetting phenomenon in this regularly firing unit is directly visible In Fig 5 c—e antidromic invasion (descending arrow) occurs at different stages in the excitation cycle of the receptor However whether a high or low

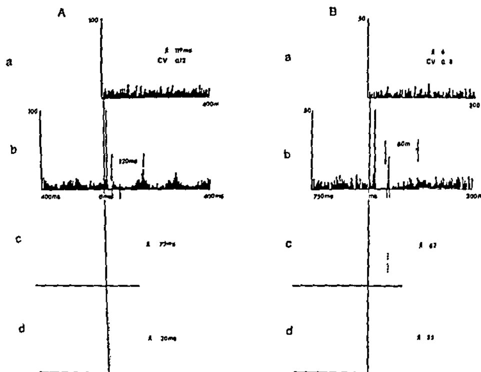


Fig 6 A & B Post stimulus events in 2 spontaneously active units a post stimulus histograms at 0V Mean impulse intervals and coefficients of variation as computed b post and pre stimulus plot at suprathreshold *en passage* stimulation Arrow Timing of antidromic invasion Mean post invasion pause in ms Bars below abscissa intervals selected for analysis in c c Time plotting of post invasion spikes (from b) with preceding spontaneous spikes Mean interval computed d as in c for spontaneous spikes occurring in time interval between stimulation and recording of orthodromic stimulation spike

probability of spontaneous firing exists at the moment of invasion an average impulse interval is added (compare coincidence in relative time of spike groupings in all three instances)

In other words the spike generating process regards an antidromic spike in the same way as it does a spontaneously occurring spike By similar analysis of post stimulus events after mechanical and DC induced orthodromic action potentials at threshold levels it was ascertained that the same resetting characteristics hold under these circumstances also

In a small minority of instances (<5 %) antidromic invasion heightened the probability of firing Two such units are shown in Fig 6 In A (b) the expected resetting (*ca* 120 ms) is visible However the period immediately following the invasion moment is evidently associated with a certain firing probability It [illegible] be argued that the antidromic spike set up by the nerve stimulation is often [illegible] by an orthodromic spike on its way from the sensory terminals This [illegible] more often in tail sensory units (of which this is an example) because of the [illegible]

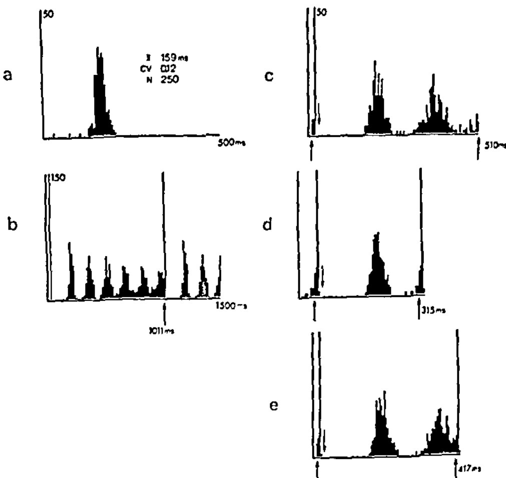


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as 100 % (left hand ordinate) The recovery curve of the nerve fibre (filled circles) is clearly more rapid than the observed resetting cycle (histogram) although a relative refractoriness of up to 30 ms is observed The recovery curve of the electrical excitability of the terminal sensory area (defined analogously from DC shocks applied to the lateral line canal in the vicinity of this unit s sensory organ) is plotted by open circles This type of stimulus presumably activates the sensory terminals in the region where spike initiation normally takes place (Murray 1956) The observation suggests that the recovery of excitability of the axon at this site is sufficiently slow to account for the recovery of spontaneous firing after antidromic invasion In other experiments a similar recovery cycle was also confirmed for the threshold to mechanical punctate stimuli at the receptor site following antidromic spike invasion

Thus it is concluded that the resetting phenomenon described above occurs in peripheral axonal branches with excitability characteristics different from the main axon

Discussion

Obviously the existence of tonic or evoked efferent activity would be of great importance for reflex regulation of the sensory cell sensitivity in the lateral line system According to Flock (1971) efferent endings of varying shape and abundance are present in all acoustico lateralis organs thus far examined except in the basilar papilla of the frog (Robbins *et al* 1967 Frishkopf and Flock 1967) However no evidence for efferent activity in the eel lateral line nerve was found even in the totally unanesthetized fish in the present investigation Possibly this could be due to a combination of the following two factors (1) The efferent activity is conducted along fibres too thin to survive peripheral nerve dissection (2) Efferent activity follows nerve fibres topographically closely associated with their afferent counterparts In this way the dissecting out of a peripheral nerve filament for recording of a set of afferents (Fig 2) will by necessity transect all efferents going to the same units The first assumption seems to hold little validity since thin afferents were repeatedly recorded from in the peripheral portion of the dissected nerve At least therefore the bimodal myelinated nerve fibre calibre distribution does not reflect a division between afferents and efferents as is the case in the mammalian ear (Galambos *et al* 1950 Fex 1962) As mentioned (Alnæs 1972 a) the nerve also contains unmyelinated fibres as do other fish lateral line nerves (Flock 1965 Roberts and Ryan 1972) All experiments describing efferent inhibitory activity on lateral line receptors have shown such activity to be conveyed through myelinated nerve fibres (Russell 1968 Hashimoto *et al* 1970 Roberts and Russell 1970) At present no discrete function has been unequivocally ascribed to the unmyelinated nerve fibre component although it may be connected with activity relating to the secretory cells and/or blood vessels in the neuromast However the possibility of an efferent inhibitory influence remains open The second assumption cannot be unequivocally dismissed However in the peripheral nerve thick and thin fibres are often seen to

be eparated in different fascicles (Fig 1 a Alnæs 1972 a) Also the recordings per formed in peripherally uncurarized fishes would presumably have detected efferent modification of sensory organ discharges The possibility remains that the efferent nerve terminals are curarized although no discernible curare effect can be detected at peripheral motor end plates and/or that the peripheral ischemia immediately blocks efferent synapses although no changes are observed in afferent activity during the first 30—45 minutes It is interesting that Katsuki and his collaborators (Katsuki *et al* 1968 Hashimoto *et al* 1970) in spite of much effort and in spite of claiming efferent inhibition in *Lyncozymba nystromi* and *Astroconger myriaster* have been unable of detecting efferent activity and/or inhibition in the japanese eel (*Anguilla japonica*) Also Yamada (1969) in a comparative anatomical study of lateral line organs of lower vertebrates reports the absence of vesiculated nerve endings in contact with the hair cells of the common eel

The experiments conducted in this study on the effects of peripheral antidromic invasion on sensory cell activity are in general agreement with those reported by Harris and Milne (1966) and Harris and Flock (1967) The evidence given by the latter for a delayed efferent inhibition (their Figs 14 and 15) seems in fact more compatible with an antidromic invasion effect than the inhibition interpretation given by these authors If the nerve was stimulated to elicit responses in large myelinated fibres an excitation of unmyelinated fibres of 0 5 μm diameter would more over have required large supramaximal stimulation intensities Their Fig 15 D similarly shows an effect rather like Fig 6 A and B of the present paper in which post invasion period is connected with increased firing probability Such effects are rarely seen as discussed above and may be due to a faulty peripheral spike in ation as hypothesized in the present paper Alternatively this backfiring effect may be caused by peripheral ephaptic or synaptic depolarization of neighbouring afferent terminals analogous to the ganglionic coupling potentials (Alnæs 1972 c) It is not known however whether afferent or efferent axons make synaptic contacts between themselves (Flock 1967)

After the study by Harris and Flock (1967) the efferent myelinated nerve fibre system in *Xenopus* was however clearly demonstrated by Russell (1968) Roberts and Ryan (1971) have similarly reported convincing functional and morphological observations on an efferent inhibitory system in the dogfish (*Scyliorhinus*) In addition an efferent inhibitory system has been reported by Katsuki s group in the japanese sea eel *Astroconger myriaster* (Hashimoto *et al* 1970) This inhibition appears to be similar in many respects to the phenomena induced by antidromic invasion A resetting of a regularly firing unit is visible in their Fig 2 and the effects of antidromic high frequency trains (Fig 3 b in the present paper) mimic the effects of presumed efferent volleys in Hashimoto *et al* 1970 (Fig 5) Moreover these authors present no data on stimulation thresholds and/or conduction velocities of presumed efferents and no checking against unwanted antidromic invasion was carried out Stimulation of dissected nerve filaments in a saline solution (see Methods in Hashimoto *et al* 1970) readily induces co-stimulation of filaments on the registra

tion electrode in the same aqueous solution as can easily be checked In fact Fig 2 of these authors actually shows the recording of the intended efferent volley in the afferent filament proving that such unintentional antidromic invasion did indeed occur

The experiments on antidromic invasion of sensory terminals have been necessary for two separate reasons *En passage* nerve stimulation can give rise to faulty interpretations of peripheral mechanisms as discussed in relation to Hashimoto *et al* (1970) Further the ensuing blocking of afferent nerve activity for a certain period after such stimulation can give rise to faulty interpretations of the response pattern observed in central higher order neurones in the afferent pathway (see Alnæs 1972 b)

With regard to the evidence presented above and in spite of the strong phylogenetic evidence for lateral line efferent inhibition the present author concludes that such activity cannot be found to play a discernible role in his preparation If this conclusion is correct the analysis of central nervous processing will probably be simpler in this system than for the lateral line systems with extensive peripheral inhibition But the problem why some species have inhibitory lateral line efferent fibres and others have not remains intriguing

The true picture would seem to be one of quantitative rather than qualitative gradations After submission of the present paper Y Yamada and K Hama (Z Zellforsch 1972 *124* 454—464) have published an EM study of the lateral line organ of the japanese eel (*Anguilla japonica*) Efferent synaptic structures are found to be very scarce in the trunk lateral line in contrast with the abundance in other fish species However head lateral line organs in the common eel display a more normal efferent innervation pattern (Flock personal communication)

The continuous support and help by Dr J K S Jansen is gratefully acknowledged Computer programming would not have been possible without constructive guidance by Dr O Sveen and excellent technical assistance was given by Mr J Rausandaksel This work was supported by the Norwegian Research Council for Science and the Humanities

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Acta physiol scand 1973 88 49—61

From the Institute of Neurophysiology University of Oslo Norway

Lateral Line Input to the Crista Cerebellaris in the Eel
Field Potentials and Histology

By

EGIL ALNÆS

Received 24 July 1972

Abstract

ALNÆS E *Lateral line input to the crista cerebellaris in the eel Field potentials and histology* Acta physiol scand 1973 *88* 49—61

The central nervous effects of electrical stimulation of the Nn lat posteriores have been investigated Maximal field potential amplitudes were found in the medial part of the ipsilateral crista cerebellaris The field potential evoked from stimulation of fast conducting nerve fibres consisted of an afferent pre synaptic volley and a post synaptic complex wave The latter is shown to consist of a population EPSP and a population spike in subcristal neurones Excitatory activity from the contralateral nerve impinges on the same neurones and bilateral summation occurs The contralateral field potential reverses more superficially than does the ipsilateral field Post excitatory reduction of population spike can be explained by refractoriness The crista region was investigated with conventional light and electron microscopical methods Myelinated primary afferent axons from the R lat post N X terminate in the subcristal region on the ipsilateral side Subcristal cells have dendritic ramifications in the molecular layer Unmyelinated axons in this layer partly consist of lateral line primary afferents These axons differ from the cerebellar parallel fibres by their extensive branching and irregular directional architecture Fusiform swellings of these axons establish abundant isomorphous synapses with dendritic spines The distribution of field potential negativity in depth corresponds to the distribution of subcristal neurones

The primary afferent neurones of the teleostean posterior lateral line system have their cell bodies located in an intracranial ganglion of the R post lat N X The cells are pseudounipolar and the central axons enter the lateral wall of the medulla oblongata caudally to the VIIIth nerve at the mid level of the fourth ventricle (Herrick 1899 Pearson 1936 Flock 1965 Alnæs 1972 a) The acousticolateral area varies greatly in extent and form in different teleosts (Larsell 1967) Most often separate nuclei can be discerned in the grey substance underlying the crista cerebellaris of the medulla oblongata They represent the terminal regions for the anterior lateral line the posterior lateral line and the acoustico vestibular input respectively The posterior lateral line lobe is continuous anteriorly with the eminentia granularis of the postero lateral lobe of the cerebellum Primary afferent fibres from the posterior lateral line nerve branch in the medulla to terminate in the grey matter

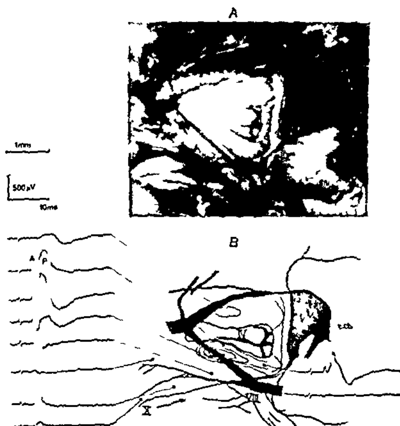


Fig 1 A *Lateral line region of the medulla as viewed through the dissecting microscope* The cerebellum is tilted rostrally *(to the right) to* expose the fourth ventricle and the adjoining cristae cerebellares B Region as in A (c cb corpus cerebelli IV fourth ventricle VIII N statoacusticus N R lat *dors* N X) *Surface* field potentials elicited by peripheral stimulation of the latter nerve are shown with *points of registration* A afferent nerve fibre volley P Synaptic response Each field potential is *computer averaged* over [illegible]0 sweeps at supramaximal nerve stimulation (1 /s) Isopotential curves of maximal P positivity drawn from several experiments

of the crista cerebellaris and bilaterally in the cerebellar postero lateral lobes and the valvula (Tello 1909 Pearson 1936 Ariens Kappers 1947 Larsell 1967) In the larval carp Tello (1909) also reported contralaterally crossing fibres at the medullary level Secondary lateral line fibres originating in the lateral line lobes pass to the same parts of the cerebellum as do branching primary fibres (Schnitzlein and Faucette 1969) and Ariens Kappers *(1947) reported* a crossed descending motor tract from large cells bordering the crista cerebellaris

The crista cerebellaris has received little attention from anatomists and physiologists despite the numerous investigations of cerebellar and lateral line mechanisms The resemblance in histological structure between this area and the cerebellum proper is mentioned by several authors, e g Houser (1901) *Ariens Kappers et al*

(1936) Larsell (1967) and Nicholson Llinas and Precht (1969) the latter authors coining the term proto cerebellum for the lateral line lobe However numerous questions arise from the interpretations of the authors mentioned above viz the submolecular cells resembling Purkinje cells or Golgi cells the origin of the fibres in the molecular layer the presence or absence of climbing fibres and/or granule cells in this area etc

In 2 previous papers (Alnæs 1972 a and b) the activity of eel lateral line afferents has been investigated The present work was undertaken to correlate the morphology and physiology of the primary afferent input to the lateral line lobe In a further report (Alnæs 1972 c) a description will be given of the impulse activity of cells within this region

Methods

The acoustico-lateral areas of the medulla oblongata of the common eel (*Anguilla anguilla*) were exposed in spinalized and/or curarized fishes (Alnæs 1972 a) Field potentials set up by stimulation of the lateral line nerve were recorded by microelectrodes in the lateral line lobes 3M NaCl electrodes of 2—7 MΩ resistance were used throughout the experiments the analogue signals from the cathode follower output being averaged by an on line Nord 1 computer Light microscopy was performed on Nissl stained and Bouin Bodian stained serial sections of 10 μm thickness throughout the entire brain in three planes Electron microscopy was carried out on specimens fixed in 2.5 % glutaraldehyde/4 % paraformaldehyde buffered to pH 7 4 (cacodylate buffer) embedded in TAAB Embedding Resin postfixed with osmium and stained with lead

Results

I Topography and surface field potentials

Fig 1 A shows the acousticolateral area of the medulla oblongata as viewed through the dissecting microscope The cerebellum is tilted rostrally (to the right) to expose the fourth ventricle The right (bottom of figure) vertical semicircular canal is similarly pushed aside to uncover the incoming R post lat N X and the VIIIth nerve complex on this side The crista cerebellaris appears as the caudal rostral ovally shaped area abutting medially to the ventricle It is obliquely traversed by a rather prominent vessel and the transversal endolymphatic duct When viewed tangentially as towards its vertical medial boundary the crista is seen to be transparent and its microcirculation could here readily be assessed Experiments were terminated when the circulation became sluggish

In Fig 1 B the surface field potentials evoked from *en passage* stimulation of the right side trunk lateral line nerve are shown from a number of recording sites The nerve stimulus was supramaximal (40 * threshold) to ensure total nerve stimulation and each trace represents an on line average of 20 sweeps The dominant surface response is characterized by a rather sharp positive negative wave (A) followed by an extended positive negative positive wave (P) The A wave corresponds in time to a mainly negative inflection in the neurogram (lower 2 records) and represents the impulse activity of the primary afferent fibres as will be shown in the following

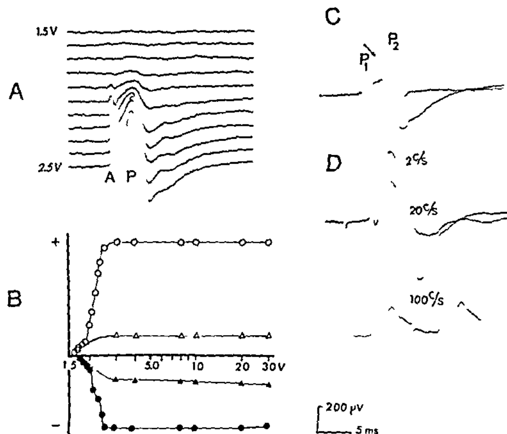


Fig 2 A Surface field potential of maximal cristal response region Ipsilateral nerve stimulation at increasing intensities through the threshold range of large calibre afferents Average of [illegible]0 sweeps B Open symbols amplitude of surface P complex positivity Filled symbols amplitude of surface I complex negativity Circles ipsilateral response Triangles contralateral response Abscissa nerve stimulation intensity (logarithmic scale) Ordinate linear scale C Averaged surface field response of rostral registration point superimposed on corresponding differentiated recording Arrow marks P_1—P_2 break 2 V stimulus 1 c/s D As in C at [illegible]0 V nerve stimulation [illegible] c/s and 20 c/s. Lower record 100 c/s

The P wave is more complex and will be shown to arise from postsynaptic events in the crista The contralateral crista response occurs with an added latency of *ca* 0 8 ms Its A wave is less clearly defined often consisting of a low amplitude ripple The shape of the P complex is similar to its ipsilateral counterpart having however, only 15—20 % of its amplitude The maximal positivity of the P complex was measured on a surface grid of 40 points (5 transversal rows of 8 points) and *iso* potential curves were drawn in three complete experiments The result is shown somewhat diagrammatically in Fig 1 B The area of P complex activity extend longitudinally along the medial half of the crista cerebellaris from a caudal level somewhat posterior to the N X medullary junction extending rostrally towards the cerebellum The field falls off sharply medially and laterally Ipsilateral and contra

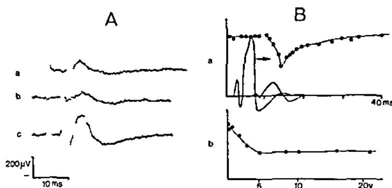


Fig 3 A Aligned surface field potentials evoked by infra maximal nerve stimulation intensities Computer averaging of 10 sweeps at 1 5 c/s a stimulation of ipsilateral nerve b stimulation of contralateral nerve c coincidence in time of a and b B a Maximal positivity of ipsilaterally evoked P complex plotted at various timings relative to (stationary small) response elicited from contralateral nerve (responses schematically outlined on curve arrow indicates relative displacement of large ipsilateral response along the abscissa) b Amplitude of P complex at 14 ms (from record a) plotted as function of contralateral nerve stimulation intensity

lateral maximum fields were found to be symmetrically situated However it was not possible to discern a marked contralateral rostral extension As can be seen from Fig 1 B the shape of the ipsilateral P-complex changes rostrally No surface positive potentials were visible in adjoining ipsi or contralateral nerves (Fig 1 B) *The slow positivity recorded in the N X ganglionic region will be discussed in a* subsequent paper (Alnæs 1972 c)

II Field potential analysis of crista response

In Fig 2 A the ipsilateral surface field potentials are shown at nerve stimulus intensities corresponding to the threshold range of large nerve afferents

The A wave which corresponds in time with the low threshold peak in the neurogram reaches its maximum within this intensity range It occurs with a latency corresponding to the conduction velocity of the large fibre group (mode at 25 m/s) and is not reduced in amplitude by nerve stimulation at higher frequencies (Fig 2 D) Furthermore it survives central nervous hypoxia for several hours By registration in depth throughout the acousticolateral area it can be seen to occur earlier at the lateral than the medial part of the ipsilateral crista and earlier in depth than at the surface (Fig 4 A) The A wave is thus interpreted as action potential activity in the primary afferent large fibre component of the N lat post

The surface positive negative positive P complex similarly reaches maximal amplitude (Fig 2 B) in the threshold range of the large primary afferents However the recruiting of high threshold thin afferents brings about a broadening of the surface P complex positivity (Fig 2 C and D (2 c/s)) Central nervous hypoxia *induced complete disappearance of the P complex within the first half hour*

Differentiation of the P complex record (Fig 2 C) shows the rising phase of the surface positivity to be diphasic (P_1 & P) Sometimes several ripples could be seen

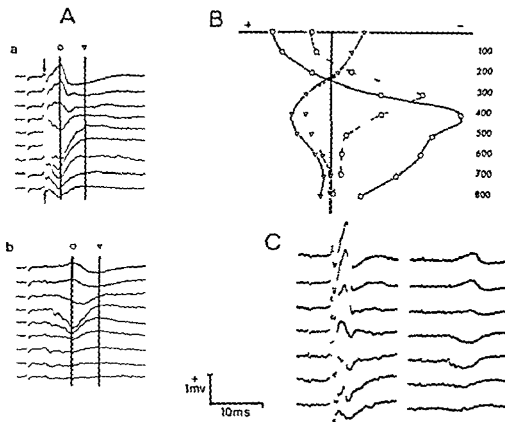


Fig 4 A a ipsilateral field responses at depth increments of 100 µm Computer averaging of 50 sweeps at 1 c/s Note earlier arrival of afferent nerve fibre volley in depth (arrows indicate same moment in time relative to stimulus) b as in a for contralateral response B Depth profiles for potentials at iso-chronous lines drawn in A Broken line contralateral responses. Depth indicated in µm C Ipsilateral (left) and contralateral (right) responses recorded at 100 µm depth increments during the same vertical penetration Note difference in reversal levels

in the P wave (Fig 4 A) The P wave (positivity and ensuing negativity) is frequency sensitive a significant reduction being visible after a few seconds of 20 c/s nerve stimulation (Fig 2 D) The remaining P_1 wave (Fig 2 D 100 c/s) is visible as a pure surface positivity of about 10 ms duration

Interaction between the responses elicited from the contra and ipsilateral nerves gave further information on the P wave and its two components In Fig 3 A the ipsilateral nerve stimulus was carefully adjusted to produce an almost pure P_1 wave (little or no ensuing negativity) The contralateral stimulus was similarly infra maximal for low threshold nerve fibres

The field response elicited from contralateral nerve stimulation (F wave ripple This is interpreted as several discrete volleys of afferent area Such asynchrony might be caused either by differences in t and/or by an added synaptic relay in the pathway The ripple stimulation frequencies and the former explanation therefore appears

b) displays an tentials in ibre vari also at his

When the two responses coincided in time (Fig 3 Ac) the resulting P wave was larger in amplitude than the algebraic sum of the 2 individual responses and a marked later negativity was added to the complex This summing effect was visible when the ipsilateral input led the contralateral by less than 10 ms

Taken together these observations suggest that the early (P_1) component of the P wave results from synaptic current induced by the afferent volley and that the P component is due to subsequent impulse activity of the same neurones This interpretation was supported by experiments in which both lateral line inputs were made maximal (Fig 3 B) A small amplitude summation was observed when inputs arrived simultaneously onto the econdary neuronal population as a measure of the increased synaptic current flowing in this case However when the maximal (P_1+P) ipsilateral response trailed the contralateral a marked reduction in the positive negative amplitudes (P) of the ipsilaterally induced P complex was observed for a period up to 25 ms Contralateral input is thus seen to enhance the synaptic current field response (P_1) but to block the spike activity (P) generated by an ensuing ipsilateral volley The latter effect may be due either to refractoriness following spike activity set up by contralateral nerve stimulation or to inhibitory synaptic activity A study of single unit activity (Alnæs 1972 c) suggests that both mechanisms are operative In Fig 3 Bb the blocking effect of the preceding contralateral volley is plotted as a function of contralateral nerve stimulation intensity A recruiting effect within the threshold range of large primary afferents is observed

Fig 4 A shows the field potentials recorded at various depths from the crista surface within the area of maximal surface positivity This positivity reverses ipsilaterally at a depth of about 250 μm to reach maximal negativity at about 400 μm In the layer 250 μm—500 μm extracellular action potentials induced by nerve stimulation were regularly picked up (Alnæs 1972 c) The P_1—P discontinuity observed at the surface coincides in time with the onset of this spike activity and so does the occasional ripple observed in the P wave The cristal areas displaying maximal surface positivities were always found to overlie those displaying maximal negativities in the depth

Changes in maximal field negativities at about 400 μm depth induced by combinatory ipsilateral and contralateral inputs also parallelled the changes described for the surface field responses

Contralateral potential reversal in depth was found to occur at more superficial levels of the crista molecular layer than that observed for the ipsilateral response In Fig 4 A reversal level and depth of maximal P complex negativity are both approximately 100 μm above the corresponding ipsilateral levels This experiment was made with contralateral electrode penetrations rendering a depth comparison somewhat insecure Stimulating alternatively from the two lateral line nerves with comparison of evoked potentials from different levels of the same track did however confirm this finding (Fig 4 C) With a depth analysis accuracy of less than 50 μm potential reversal was found to occur 150—200 μm superficially on the contralateral side Thus it is tentatively concluded that contralateral synaptic activity is distributed

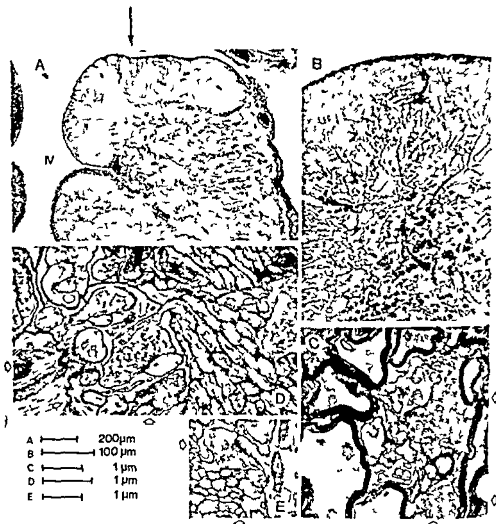


Fig 5 A Bodian stained transversal section through the crista cerebellaris IV fourth ventricle Arrow marks microelectrode penetration direction through molecular layer In the lateral part of the submolecular region a multilayered pattern of lateral line nerve axons is visible B Bodian stained transversal section through medial part of the crista cerebellaris at higher magnification Neurones in the submolecular layer have dendritic ramifications in the overlying strata Myelinated axons cut at all angles in the region of cell somata C EM from submolecular layer Note profusion of myelinated axons Two presynaptic structure (arrows) containing pleomorphous vesicles are visible The upper structure is seen to contact a dendritic process D—E EM s from lower part of molecular layer Thin unmyelinated axons containing rounded vesicles are cut at various angles A number of isomorphous pres naptic structures are visible These originate from the unmyelinated axons (D arrows) and contact dendritic spines (E arrows)

further towards the crista surface on the dendritic tree of subcristal neurones than is the ipsilateral input

Due to ... ow stimulation range for full P complex inducement it was only occasional... to obtain pure and graded P_1 positivities at low threshold/lod

frequency nerve stimulation Usually a superposition of P_1 and P waves was manifest even at low stimulus intensities A mirroring amplitude symmetry between surface P complex positivity and negativity was evident throughout the intensity range (Fig 2 B) Similarly the depth reversals observed for ipsilateral P complexes displayed a mirror like symmetry for the maximal positive and negative surface amplitudes (Fig 4 B) Thus it appears reasonable to regard the P -component as a coherent and essentially triphasic wave induced by the synaptic current giving rise to the P_1 diphasic wave The triphasic P wave is tentatively concluded to originate from the action potential activity and ensuing hyperpolarization induced in sub cristal neurones

Contralateral depth profiles were sometimes found to be slightly positive negative asymmetric (Fig 4 B) This probably suggests a more complex genesis of the *contralateral synaptic response The intensity/P amplitude plot (Fig 2 B) and* single unit analysis of these cells show that many second order neurones are activated at low levels of nerve stimulation (Alnæs 1972 c) At higher levels of nerve stimula tion afferent activity and synaptic processes originating in higher threshold afferents will make some contribution to the P response extending the surface positivity in time (Fig 2)

Penetration of subcristal cells was successfully accomplished only in a very limited number Rapidly deteriorating membrane potentials of $\leqslant 40$ mV amplitudes were recorded with 2 M KCl electrodes of 30 MΩ resistance In the best cells a large EPSP of 15 ms duration was elicited by ipsilateral nerve stimulation The EPSP followed frequencies above 20 c/s It corresponded in time to the P_1 wave and this observation supports the interpretation given above on the origin of this part of the field response No evoked or spontaneous IPSP s were detected in these experiments spontaneous EPSP s were however present

III Histology

The entire acoustico lateral portion of the medulla oblongata was investigated by conventional light and electron microscopical methods In serial Bodian sections the incoming large calibre primary afferent nerve fibres were found to curve dorsally along the lateral wall close to the surface of the medulla in a rostral direction

Extensive branching of single axons occurs dorsally often without apparent di ametre reduction and transversal and rostral axonal fascicles develop in a multi layered pattern (Fig 5 A) In horizontal sections axons in the rostral fascicles are seen to give off medial branches even at the most rostral level of the acoustico lateral area Large calibre transversal axons can be followed medially where they branch and undergo diametre reduction in the layers immediately above and below the somata of the subcristal neurones Caudally to the nerve medullary junction fascicles of thin axons *are seen to cross the midline below the ventricle floor However these axons* could not be continuously traced to lateral line afferents Subcristal neurones are aggregated medially at a depth of 250—500 μm from the medially curved cristal surface (Fig 5 A) The cell bodies are of medium size (max width < 15 μm) and have a fusiform to multipolar shape Thick dendrites can be followed radially into the overlying molecular layer where they undergo extensive branching (Fig 5

Rostral cells have wide flask shaped axonal hillocks and are more densely aggregated than caudal neurones. Granule like cells are absent below the molecular layer in the whole caudal rostral extension of the crista cerebellaris. The molecular layer appears virtually devoid of cells in the light microscopy preparations save for dendritic arborizations and a rich capillary network. At the mid nerve rostro-caudal level this layer extends to a depth of 250 μm from the surface. There is a very sharp transition from the unmyelinated molecular layer structure to the underlying zone of intermingled dendrites and myelinated thin axons.

In the EM the submolecular zone is characterized by a plexus of mitochondria rich myelinated axons (Fig 5C). The axons range in diametre from 2.0 to 0.5 μm and display a continuous variation in the number of myelin lamellae. The density of axons is highest immediately below the somata of the subcristal cells, often clusterings of myelinated axons are visible around the initial axonal segments of these cells. Very few synaptic structures are found touching these portions of the cell membrane. However some scattered boutons are found in synaptic contact with narrow dendritic processes. These processes are similar in structure to the dendrites observed in the molecular layer. The majority of submolecular boutons are of the type later to be described in the molecular layer containing isomorphous rounded vesicles. A limited number of presynaptic elements in this layer however contain pleomorphous vesicle aggregations (Fig 5C).

The subcristal cells reveal a fusiform branched soma shape with wide dendritic processes (3—4 μm in diametre) extending towards the surface. The myelinated axons now of < 1 μm diametre surround the initial part of these processes but only a few synaptic structures are present in the somatal and immediate suprasomatal layers. There is an abrupt decline in axonal diametre and degree of myelinization as one proceeds dorsally. Simultaneously a profusion of densely packed unmyelinated axons appears (Fig 5D). These axons are of < 0.15 μm diametre and can be seen to undergo extensive branching in all directions throughout the entire height of the molecular layer. Bundles and fascicles of such axons are often cut to varying degrees of obliqueness within the same section creating a whorl like picture. These axons contain rounded vesicles throughout their course.

Homogenous dendritic contours are observed throughout the molecular layer, all being of the type associated with the subcristal cells. Extensive gradual tapering occurs towards the cristal surface, dendrites of about 1 μm diametre are however observed even at the most superficial levels. A few glial cells are concentrated towards the surface. Clearly defined spines are abundantly present on all dendritic branches. They appear as elongated rounded structures of about 0.5 μm diametre often connected to the dendrite by a narrow stalk of up to 1 μm length (Fig 5E).

All synaptic structures in the molecular layer are homogenous in type, fusiform axonal boutons making asymmetric synaptic contact with dendritic spines. No synapses are found on dendritic stems. The presynaptic terminals can regularly be seen to arise from swellings of unmyelinated axonal branchlets. They commonly attain a diametre of 0.6—0.8 μm and contain several mitochondria and a concen

tration of the isomorphous rounded vesicles also found throughout the axons (Fig 5 D and E) In one instance the transition from submolecular myelinated axon to molecular unmyelinated axon giving rise to typical bouton could be traced in continuity

All synapses are morphologically unidirectional and no dendro axonic axo-axonic or dendro dendritic synapses were found Apart from the relative absence of spines and hence the synaptic scarcity observed on first order dendritic branches no quantitative differences in synapse numbers are readily visible as function of depth

Discussion

The field potential analysis of the present study has been useful for determining the area of maximal synaptic activity and for identifying the elements generating the synaptic current resulting from lateral line activity The area corresponds to the crista cerebellaris as defined anatomically molecular layer dendrites of submolecular neurones serving as current generators The postsynaptic component of the ipsilateral field response exhibits an early phase (P_1) ascribed to excitatory synaptic currents Its depth profile is determined by the distribution of active synapses on the dendrites superficial dendritic branches acting as sources for transmembrane depolarizing current sinks in the deeper layers The more superficial reversal level of the contra lateral response suggests a more peripheral extension of these synapses Alternatively a more pronounced ipsilateral spike response would shift the ipsilateral isopotential curve to deeper layers A precise analysis of synaptic topography from laminar field potential analysis alone does not seem possible in this structure The cell bodies of the subcristal neurones are not located at a uniform depth from the surface but are aggregated at 250—500 μm depth from the medial cristal curvature The cyto architecture of these cells and the afferent terminals is irregular and complicated Also the transversal/horizontal dimension of the subcristal neurone population is small compared with its vertical dimension These factors contribute substantially into obscuring the resultant field activity (Humphrey 1968) Clearly the crista does not constitute a homogenous extended laminated structure to which the theory of synchronous activity in a parallel core conductor system can be unequivocally applied It remains for morphological studies of degenerating afferent fibres to elucidate the presynaptic terminal pattern For the same reasons pronouncements on the possibility of spike propagation in dendritic branches (Llinas and Nicholson 1971) are conjectural in the absence of intradendritic recordings

The late phase (P) of the field response has been ascribed to impulse activity within the same population of neurones The present experiments indicate that both ipsilateral and contralateral inputs produce a high degree of synaptic activity on subcristal neurones

The symmetry observed between (surface) positivity and ensuing (surface) negativity of the P wave both for increasing stimulation intensities and as regards their

depth profiles are arguments against a substantial forward or recurrent inhibition being responsible for the late P phase Nicholson *et al* (1969) have reported preliminary evidence for recurrent inhibition of more than 150 ms duration in the lateral line lobe of the electrosensitive lateral line system of an elasmobranch In the eel even primary nerve afferents display relative refractoriness ⩽ 25 ms (Alnæs 1972 b) and the prolonged depression reported by Nicholson *et al* (1969) is not observed Single unit analysis of subcristal neurones (Alnæs 1972 c) confirmed refractoriness to be the cause of ipsilateral post activity suppression Stimulation of the contralateral nerve yields a mixed response of excitatory effects and inhibition of less than 25 ms duration

The crista cerebellaris has been named from its similarity to the cerebellar cortex proper (Houser 1901) *This similarity has recently been re emphasized by Nicholson et al* (1969) who coined the term proto cerebellum The present investigation suggests that primary afferent fibres establish direct synaptic contacts on dendritic spines of submolecular neurones This is in agreement with Larsell (1967) who reported terminal lateral line nerve axons in the molecular layer of *Catostomus* in Golgi and reduced silver preparations Moreover the unmyelinated axons observed throughout the molecular layer appear to be different from cerebellar parallel fibres for three reasons (1) The axons of the molecular layer are partly or largely primary afferent fibre and cells analogous to the cerebellar granule cells are not present in the subcristal layers (Such cells have however been reported in other preparations (Nicholson *et al* 1969) especially in the rostral parts of the lateral line lobes) It seems unlikely that the fibres originate in the continuous but distant (> 2.5 mm) cerebellar cortex proper (2) A very different cytoarchitecture from that of the cerebellum is observed fibre fascicles course in convoluted patterns (3) The unmyelinated fibres branch profusely and the synaptic terminal is often seen to arise from a T branchlet of the molecular parent axon

The signal processing of the crista cerebellaris is obviously more complicated than implied by the present study The origin of the few pleomorphous presynaptic terminals on submolecular dendritic processes remains to be elucidated and it must be emphasized that anterior lateral line and acoustico vestibular inputs are also fed into the molecular fibre network Their effects have not been studied in the present investigation However the conclusion is reached that the inframolecular cells represent true secondary neurones in the eel lateral line pathway and will therefore be the first central nervous neurones to be activated by incoming lateral line activity Other secondary cells obviously exist rostrally to these but no major field potentials were picked up in a preliminary survey of the cerebellum (Alnæs unpublished) Other authors reporting on central nervous neurones in the lateral line system (Enger and Szabo 1965 Nicholson *et al* 1969) have been unable of specifying the central nervous synaptic pattern and/or relating higher order activity to that of primary afferents This situation seems clearer in the crista cerebellaris of the eel and will be reported on in a subsequent paper (Alnæs (1972 c)

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Redistribution of Tissue Histamine Stores (Basophil Leucocytes) of Turtles in Response to Submersion and Cold Exposure

By

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Abstract

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The histamine contents of liver and blood of turtles (*Pseudemys scripta elegans*) kept at 20—[illegible] °C were compared with those of turtles exposed to cold (4—[illegible] °C) for 2—21 days or submerged for 1[illegible] hours. Submersion and cold exposure consistently caused a decrease in the histamine content of blood and an increase in the histamine content of liver. The average histamine content of blood was reduced to about half its pre-exposure value and that of liver was doubled. The results indicate that the basophil leucocytes, which are exceptionally numerous in this species and are the only blood cells known to contain appreciable amounts of histamine, accumulate in the liver when the turtles are submerged or exposed to cold. Influence of cold exposure on the histamine content of liver and blood was also studied in several other species of turtle (*Chrysemys picta, Emys orbicularis, Clemmys caspica leprosa, Terrapene carolina, Kinosternon flavescens*) and they all showed a response similar to that observed in *Pseudemys scripta elegans*.

Tissues with secretory functions may contain a large proportion of non-mast-cell histamine, but otherwise mast cells and basophil leucocytes constitute the major histamine store in vertebrates (Reite 1972). Since basophil leucocytes are scarce in most mammalian species, the histamine stores of mammals are mainly confined to tissues rich in mast cells. The physiological importance of mast cell histamine has been doubted (Kahlson and Rosengren 1968).

Many turtles, especially fresh water forms (terrapins), have high numbers of basophil leucocytes and migration of basophils into the extravascular tissues is evident (Michels 19[illegible], Reite 1970). Thus, contrary to mammalian histamine stores, those of turtles are mobile. The histamine stores of turtles could be redistributed by diverting the basophil leucocytes towards particular tissues. However, it has not

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been demonstrated that redistribution of the histamine stores of turtles actually does occur Seeking an answer to this question I have undertaken a study of the effect of environmental changes on turtle tissue histamine

Materials and methods

Red eared turtles (*Pseudemys scripta elegans*) weighing 200—300 g obtained from an animal dealer in Wisconsin U S A were the principal experimental animal They were kept at temperatures of 20—22 C in tanks with shallow water and were fed minced meat and liver once a week No food was given during the last week prior to an experiment Turtles maintained in the laboratory as indicated above served as controls whereas other turtles were placed in a room holding a temperature of 4—6 C for periods of 2—21 days (continually in shallow water) and still others were submerged for 12 h (water temperature 20—22 C) Each group consisted of 7 animals and the experiments were performed in the period from May to August both months inclusive To obtain tissue for determination of histamine content the turtles were removed from the water and stunned by a blow on the head After rapid drying of the neck with a towel the carotid artery was cut and blood collected in a test tube previously flushed with a dilute solution of heparin The turtles were then decapitated the plastron removed and samples were taken from liver and other tissues as required Histamine was assayed fluorometrically according to the procedure of Shore Burkhalter and Cohn (1959) except that citric acid was used instead of hydrochloric acid in the final step of the assay (to stabilize the fluorophore) The method of Juhlin and Shelley (1966) was applied for histochemical localization of histamine

Experiments involving cold exposure were also performed in a few specimens of other species of turtles (*Chrysemys picta* *Emys orbicularis* *Clemmys caspica leprosa* *Terrapene carolina* and *Kinosternon flavescens*) all with body weights of 100—400 g Young turtles of the species *Chrysemys picta* and *Pseudemys scripta elegans* were used for determination of whole body histamine content Relation between liver histamine and histamine in the rest of the body (except the shell) was studied in young specimens of the latter species only The young turtles weighed 7—9 g All experiments were carried out in Oslo

Results and discussion

Initial studies of 3 animals from each of the main groups of *Pseudemys scripta elegans* included determination of histamine in blood liver skeletal muscle skin lung oesophagus and intestine The histamine levels in the 5 latter tissues did not seem to show significant group differences Further experiments were therefore limited to assay of histamine in blood and liver which revealed a consistent increase in the histamine content of the liver compared to the histamine content of blood in both submerged and cold exposed turtles (Table I) In turtles kept at low temperatures this change had occurred already after 2 days and no further change was observed in those killed after 2—3 weeks Individual histamine levels are the mean of results obtained by parallel assay of 2—3 one ml samples of blood and 3—5 samples (each 0 5—1 g) taken from different parts of the liver The difference in histamine contents of blood samples from the same animal were slight but a somewhat larger difference was found between liver samples Assay of the remainder of the liver in one turtle from each group showed however that the mean of the histamine content of the small samples was a good indicator of the histamine content of the liver as a whole Histochemical observations of liver smears after staining with o phthalaldehyde and subsequent staining with toluidine blue showed that basophil leucocytes and tissue mast cells were the only cells where presence of

TABLE I. Effect of submersion and cold exposure on histamine content of blood and liver of the red-eared turtle (*Pseudemys scripta elegans*). Histamine levels are expressed as μg base/ml for blood and as μg base/g wet tissue for liver. Individual values within each group are arranged according to histamine levels found in blood

Individual or average values	Control turtles kept at 20–22° C		Turtles submerged for 12 h		Turtles exposed to cold (4–6° C)	
	Blood	Liver	Blood	Liver	Blood	Liver
Individual	7.8	4.7	6.2	22.9	4.3	22.1
	8.9	9.7	6.4	17.7	4.5	21.4
	11.3	10.0	8.3	35.6	6.9	28.1
	12.9	18.1	8.5	39.4	7.3	31.5
	19.1	16.3	9.8	36.5	8.6	22.3
	19.6	15.3	9.9	29.7	12.2	34.6
	22.6	27.1	19.4	45.6	13.3	30.0
Average	14.6	14.5	9.8	32.5	8.2	27.1

histamine could be demonstrated. The two cell types are difficult to distinguish from each other in this turtle, and both their relative number and the density of granules within them seem to vary. It has previously been noticed that there is possibly less histamine in true tissue mast cells than in basophil leucocytes (Reite 1970). These observations may partly account for some of the individual differences in tissue levels of histamine. Small individual variations in packed cell volume (after centrifugation of blood) were noted, but such variations could not explain the variation between the groups with respect to blood histamine.

On cold exposure turtles of the species *Chrysemys picta*, *Emys orbicularis*, *Clemmys caspica leprosa*, *Terrapene carolina* and *Kinosternon flavescens* showed a similar pattern of histamine redistribution as that observed in *Pseudemys scripta elegans*. The ratio *histamine per g liver/histamine per ml blood* was 0.6—1.5 at 20—22° C and changed to 2.5—5.0 following exposure to temperatures of 4—6° C. This observation was consistent despite the fact that the absolute levels of tissue histamine varied considerably from species to species (about 10 times higher in *Chrysemys picta* than in *Terrapene carolina*, intermediate levels in the others). Assay of whole body histamine (shell included) in young specimens of *Chrysemys picta* and *Pseudemys scripta elegans* gave values of 7.0—11.3 μg/g and 6.5—9.2 μg/g, respectively.

The histamine contents of the liver and the rest of the body (except the shell) in 5 young specimens of *Pseudemys scripta elegans* kept at 20—22° C as indicated for the larger turtles of this species and in another 5 of similar size kept in a cold room (4—6° C) for 2—3 weeks are listed in Table II. The results confirm that low ambient temperatures cause accumulation of histamine in the liver.

All turtles referred to so far were studied during the summer, but several specimens of *Pseudemys scripta elegans* (b.w. 200—300 g) were also kept in the laboratory throughout the winter. Some of these refused to take food, especially during

TABLE II *Effect of cold exposure (4–6° C) on tissue histamine in young specimens of the red eared turtle (Pseudemys scripta elegans)*

Experimental group	Tissue	Histamine content (μg base/g wet tissue)	
		Range	Average
Control turtles (20–22° C)	Whole liver	8.2–20.4	13.0
	Remaining tissues except shell	9.5–17.1	12.4
Turtles exposed to cold	Whole liver	14.2–27.6	18.9
	Remaining tissues except shell	7.1–11.0	8.8

December and January, and reverted to an inactive state. Assay of tissue histamine in such turtles showed that they had a histamine distribution similar to that of turtles which had been subjected to submersion or cold exposure. It thus seems that the pattern of histamine distribution obtained during submersion and cold exposure is the normal winter pattern. Additional evidence that this is true was obtained in turtles (*Pseudemys scripta elegans*) kept in the cold room (4—6° C) for 2—3 weeks and subsequently brought back to a room with temperatures of 20—22° C. During the summer months the distribution pattern for histamine prevailing before cold exposure was re-established within 24—36 h. However, when active turtles were forced to establish the winter histamine pattern during the autumn, they tended to retain it for a longer period after removal from the cold room.

The present study provides strong evidence that both submersion and cold exposure cause accumulation of basophil leucocytes in the liver of turtles. Fresh-water turtles are exceptional among air-breathing vertebrates in their ability to survive in the submerged state (Musacchia 1959) or in an atmosphere of pure nitrogen (Johlin and Moreland 1933, Belkin 1963). They probably do this by switching to anaerobic glycolysis (Belkin 1963, 1968; Robin *et al.* 1964), which would result in an increased tissue demand for glucose if the energy requirement remained the same. It is noteworthy that some of the species of turtles presently studied have previously been shown to have considerably increased levels of blood glucose during submersion (Robin *et al.* 1964) and cold exposure (Rapatz and Musacchia 1957, Hutton 1964). The increased blood glucose levels in anoxic turtles can be accounted for by corresponding decrease in liver glycogen (Daw, Wenger and Berne 1967). Many fresh-water turtles probably spend the winter at the bottom of ponds (Carr 1952, Musacchia 1959) and are then exposed to both submersion (anoxia) and cold. Experiments are now underway to determine whether there is any connection between the increased histamine levels (accumulation of basophil leucocytes) in the liver and the increased levels of blood glucose.

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Acta physiol scand 1973 88 67—70

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On the Relative Importance of Extra Neuronal Uptake of Noradrenaline Released by Nerve Stimulation in the Rabbit Heart

By

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Abstract

JUNSTAD M L STJÄRNE and Å WENNMALM *On the relative importance of extra neuronal uptake of noradrenaline released by nerve stimulation in the rabbit heart* Acta physiol scand 1973 *88* 67—70

Drugs known to inhibit the extra neuronal uptake of noradrenaline were given to the sympathetically stimulated perfused rabbit heart and the subsequent change in the overflow of transmitter was studied Normethanephrine (10^{-5} M) increased the outflow of noradrenaline on nerve stimulation by about 25 % while lower doses were ineffectual However in the dose mentioned the drug did not potentiate the earlier reported increase in overflow caused by desmethylimipramine (5.5×10^{-7} M) On nerve stimulation intact noradrenaline made up 83 % of the increase in radioactivity in the perfusate before and 94 % after inhibition of the extra neuronal uptake of noradrenaline It is concluded that extraneuronal uptake of noradrenaline released by sympathetic nerve stimulation plays a small but significant role in the inactivation of the transmitter from the neuro-effector junctions The findings are discussed with regard to earlier observations where phenoxybenzamine another potent inhibitor of extra neuronal uptake was found to increase the overflow of transmitter on nerve stimulation more than could be expected from its uptake inhibiting properties alone

Noradrenaline (NA) released from sympathetic nerve endings is to a large extent inactivated by binding in the tissues (*cf* Iversen 1967) both in sympathetic nerve terminals and in extra neuronal tissue Extra neuronal uptake Uptake was initially observed at very high NA concentrations Recently it has been proposed (Lightman and Iversen 1969) that NA is taken up extra neuronally even at lower NA levels

In the isolated perfused sympathetically innervated rabbit heart drugs blocking uptake of NA into sympathetic nerves such as cocaine or desmethylimipramine (DMI) approximately double the amount of NA overflowing in the perfusate on nerve stimulation (Stjarne and Wennmalm 1971 Wennmalm 1971) It thus seems that normally when the uptake mechanism is not depressed about one half of the amount of NA liberated is recaptured into the neurons However phenoxybenzamine

(PBA) raised the outflow of NA on nerve stimulation three fold This additional increase in outflow of NA compared to e g cocaine was interpreted as a result of increased release of NA on nerve stimulation However since PBA in contrast to cocaine or DMI blocks extra neuronal as well as neuronal uptake of NA the greater rise in NA efflux induced by PBA might be due to inhibition of extra neuronal NA uptake

The present study was performed to clarify this issue by using normethanephrine (NMN) to block extra neuronal uptake of NA released in response to sympathetic nerve stimulation in the perfused rabbit heart

Methods

21 rabbits of both sexes and mixed strains were used for the study They were killed by a blow on the head and bled from the left carotid artery The heart with intact left and right sympathetic nerve supply was dissected out according to Huković and Muscholl (1962) The organ was perfused at a temperature of 37 C and at a pressure of about 60 cm H_2O with Tyrode's solution (conc in mM NaCl 136 9 KCl 2 7 $CaCl_2$ 1 8 $MgCl_2$ 1 0 $NaHCO_3$ 11 9 $NaHPO_4$ 0 4 glucose 5 6) aerated with 6 5 % CO_2 in O_2 Ascorbic acid 20 μg/ml and atropine (10^{-6} g/ml) was added to the solution The nerves with adjacent tissue were pulled through separate plastic tubes with platinum rings in their walls serving as electrodes and connected to Grass Mod 1 S5 stimulators They were stimulated by rectangular pulse trains of supramaximal strength and 1 ms duration Each heart was stimulated twice with a 15 min interval The stimulation frequency was 10 Hz and the number of pulses in each stimulation was 300 The apex of the heart was connected to a strain gauge transducer and heart rate and contractile force were recorded on a Grass Model 5 D Polygraph The effluent from the heart was collected during the nerve stimulations and until the contractile response had faded out After adsorbtion on alumina the effluent content of NA was assayed fluorimetrically according to Euler and Lishajko (1961) The recovery of NA was 83±6 % (n = 7) The NA values given in this paper are not corrected for the losses during the purification

In one series of experiments 16 hearts were used to study the effect of NMN or of NMN together with DMI on the outflow of NA NMN (10^{-7} M to 10^{-5} M) or NMN (10^{-5} M) and DMI (5.5×10^{-7} M) were added to the perfusion medium 10 min before the second nerve stimulation The ratio between the NA outflow during the second and that during the first nerve stimulation periods was used as an index of the capacity of NMN to affect the extra neuronal elimination of transmitter

In another series of experiments 5 rabbits were injected with 50 μC of 1 NA ^{3}H (Amersham) and sacrificed 30 min later The hearts were perfused and stimulated as described above and PBA (3×10^{-6} M) was added to the Tyrode's solution 10 min before the second stimulation After purification on alumina NA in the effluent was further purified by ion exchange chromatography (Amberlite CG 120 mesh 200—400 sodium form 4×80 mm) 0 5 ml aliquots of the alumina and Amberlite eluates as well as of the perfusate from the heart were added to 20 ml of a 7 3 toluene absolute ethanol solution containing 4 g of 2 5 diphenyloxazole and 100 mg of 1 4 bis 2(methyl 5 phenyloxazolyl)benzene per liter of toluene The radioactivity in the aliquotes was assayed in a Packard Tri Carb Liquid Scintillation Spectrometer

Results

Stimulation of the sympathetic nerves to the heart during 30 s at 10 Hz caused an immediate and marked increase in heart rate and contractile force The outflow of NA in the effluent was 239±18 ng (mean±SE n = 19) A second nerve stimulation performed 15 min later caused in an earlier control series an outflow of NA which was 88±3 % of that during the first nerve stimulation

NMN 10^{-7} M or 10^{-6} M did not significantly increase the outflow of NA during the second nerve stimulation as compared to that in a control series nor was the mechanical response of the heart changed At NMN 10^{-5} M the outflow of NA

was increased by 26 % ($P^* < 0.05$) compared to controls. NMN (10^{-5} M) together with DMI (5.5×10^{-7} M) raised the outflow of NA during the second nerve stimulation to 171±13 % of that during the first stimulation. This increase is not statistically different from that obtained in an earlier control series (Wennmalm 1971) where DMI (5.5×10^{-7} M) alone caused an increase in the outflow of NA to 206±27 % of that during the preceding control stimulation.

Stimulation of the sympathetic nerves of the heart from rabbits previously injected with radioactive NA caused a rise in the outflow of radioactive material in the effluent from the heart. In the absence of PBA 83±4 % (mean±SE, n = 5) of the increase in radioactivity in the perfusate during nerve stimulation consisted of intact NA. When PBA (3×10^{-6} M) was added to the perfusion medium, the relative amount of intact NA in the perfusate following nerve stimulation was raised to 94±7 % (n = 5). The difference is statistically significant ($P^* < 0.05$).

Discussion

The present study was performed to evaluate the relative importance of extraneuronal uptake of NA released in response to sympathetic nerve stimulation in the perfused rabbit heart. It has earlier been shown that PBA, which is an inhibitor of both neuronal (Axelrod, Hertting and Potter 1962) and extraneuronal (Iversen 1965) uptake of NA, increases the overflow of transmitter in response to nerve stimulation to a greater extent than certain other drugs inhibiting neuronal uptake alone, such as cocaine or DMI (Stjärne and Wennmalm 1971, Wennmalm 1971). Similar observations have earlier been made in studies of the isolated perfused cat spleen (Thoenen *et al.* 1964 a, b). The difference between PBA and cocaine in ability to enhance NA outflow in the sympathetically stimulated spleen might, at least in part, be due to the fact that PBA augments perfusion flow by blocking vasoconstriction, thus facilitating wash-out of NA released from the nerves. However, the same explanation is probably not valid for the rabbit heart, since the perfusion flow rate following blocking of the alpha adrenergic receptors is only moderately increased (*cf.* Wennmalm 1971). The fact that PBA raises the overflow of NA from the heart following nerve stimulation to a greater extent than cocaine suggests that PBA may increase the amount of NA released by each nerve impulse. However, since PBA has been shown to inhibit extraneuronal as well as neuronal uptake of NA (Iversen 1965), the additional overflow of NA on nerve stimulation caused by PBA compared to cocaine might result from a more complete tissue inactivation of transmitter. This does not seem likely in view of the present results, for the following reasons.

1. NMN, which has been reported to be a potent inhibitor of extraneuronal uptake while affecting reuptake into the neurons only to a small extent (Iversen 1965), raised the overflow of NA caused by sympathetic nerve stimulation by about 25 % when applied at 10^{-5} M. Lower doses were found ineffectual. The observation suggests that extraneuronal uptake of NA plays a small but significant role in the elimination of transmitter in the rabbit heart.

2 NMN (10^{-5} M) did not further potentiate the effect of DMI (5.5×10^{-7} M) on overflow of NA following sympathetic nerve stimulation There is thus not reason to assume that the larger overflow of NA seen after nerve stimulation in the presence of PBA compared to *e g* DMI results from the simultaneous inhibition of neuronal and extra neuronal uptake of NA

3 Langer (1970) has recently shown that PBA prevents metabolic inactivation of NA released on nerve stimulation In the present study 83 % of the increase in radioactivity in the perfusate during nerve stimulation in the absence of drugs was identified as intact NA Following administration of PBA which prevents metabolic inactivation of NA in extra neuronal tissue 94 % of the radioactivity in the perfusate was identified as intact NA This shows that a) the amount of NA O methylated after liberation from the nerves is limited and b) that extra neuronal metabolic inactivation and thus its prerequisite extra neuronal uptake plays a small role in the inactivation of transmitter

In conclusion the results presented here suggest that in the sympathetically stimulated rabbit heart extra neuronal uptake plays a small but significant role in the inactivation of transmitter In addition inhibition of extra neuronal uptake of NA is probably not the main explanation for the large overflow of NA following nerve stimulation seen in the presence of PBA compared to other uptake inhibiting drugs The results thus indirectly favours the hypothesis that PBA in addition to its uptake and alpha adrenergic inhibiting properties increases the release of transmitter in response to nerve stimulation (Stjarne and Wennmalm 1971 Wennmalm 1971)

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Kinetic Observations on Cholinesterase Activities of Rat Brain and Sympathetic Ganglion towards Biochemical and Histochemical Substrates

By

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Abstract

ERANKO L *Kinetic observations on cholinesterase activities of rat brain and sympathetic ganglion towards biochemical and histochemical substrates* Acta physiol scand 1973 *88* 71—83

Cholinesterase activities of homogenates of rat brain and superior cervical ganglion were measured by automatic titration at pH 8 Acetylcholine acetylthiocholine acetyl β methyl choline propionylcholine propionylthiocholine α naphthyl acetate butyrylcholine butyrylthio choline and benzoylcholine were used as substrates As selective inhibitors of specific and non specific cholinesterases 1 5-bis-(4 allyl dimethylammoniumphenyl) pentan 3-one diiodide (284 C 51) and tetra isopropylpyrophosphoramide (iso-OMPA) were used respectively Acetyl β methylcholine and α naphthyl acetate were mainly split by specific cholinesterase butyrylcholine was predominantly hydrolyzed by non specif c cholinesterase while acetylcholine acetylthiocholine propionylcholine and propionylthiocholine were readily split by both enzymes Michaelis—Menten constants determined by the Lineweaver Burk procedure were equal for acetylcholine and propionylcholine on the one hand and for acetylthiochol ne and propionyl thiocholine on the other The specific (284 C 51 sensitive) components of the cholinesterase activities of these 4 substrates showed strong inhibition by excess substrate while the non specific activity towards the same substrates increased with increasing substrate concentration When ? of these substrates were in turn used together as substrates no additive increase of activity was observed It is concluded that prop onylcholine and propionylthiochol ne serve like acetylcholine and acetylthiocholine as substrates of both specif c and non specific chol n esterases in rat brain and sympathetic ganglion

A vast literature has accumulated on the biochemistry of cholinesterases critically reviewed by Augustinsson (1948 1963) Whittaker (1951) as well as Cohen and Oosterbaan (1963) The histochemistry of cholinesterases has also been dealt with in a large number of publications reviewed by Koelle (1963) Harkonen (1964) and Pearse (1972) There is a general agreement of the existence of 2 major types of cholinesterases the specific true or acetylcholinesterase (E C 3 1 1 7) and the non specific pseudo or butyrylcholinesterase (E C 3 1 1 8) a division whose validity has been repeatedly tested and proven with a large variety of substrates inhibitors and enzyme preparations (Mendel and Rudney 1944 Augustinsson 1948 Sawyer and Hollinshead 1945 Ord and Thompson 1952 Aldridge 1953 Bayliss and Todrick

1956 Koelle 1963 Eränkö *et al* 1964 Härkönen 1964) The terms specific and non specific are systematically used in the present paper These 2 types of cholinesterases have been reported to differ also in regard to kinetic properties For instance the specific cholinesterase is inhibited by excess substrate while the activity of non specific cholinesterase increases with increasing substrate concentration (Sawyer and Hollinshead 1945 Augustinsson 1948 Myers 1953, Bergmann and Segal 1955)

The present study was undertaken to investigate the kinetic properties of cholinesterases in homogenates of rat brain and sympathetic ganglion using several biochemical and histochemical substrates together with selective inhibitors, since no systematic study is available on this subject

Material and methods

Preparation of tissues

In this study about 300 rats of Sprague Dawley strain were used The animals were killed under light ether anesthesia by cutting the vertebral column and aorta by scissors The brain and both superior cervical ganglia were removed They were then quickly weighed with an analytical balance in a weighing bottle After cutting of the tissues into small pieces they were homogenized in 0 1 M CO_2 free KCl solution in a ground glass homogenizer at 0 C The homogenates contained 100 mg/ml of brain tissue and 5—10 mg/ml of ganglion tissue To facilitate the liberation of enzyme the homogenates were frozen with solid CO_2 and thawn 5 times during the homogenization The homogenates were transferred into glass stoppered test tubes and bubbled through with CO_2 free nitrogen for 5 min The homogenates were stored frozen at −50 C The determinations of enzyme activity were carried out within 2 days during which time the cholinesterase activities were shown to be unchanged

Substrates and inhibitors

The following substrates were used acetylcholine iodide acetylthiocholine iodide acetyl β methylcholine chloride propionylcholine iodide propionylthiocholine iodide butyrylcholine iodide butyrylthiocholine iodide and benzoylcholine chloride The final substrate concentration in the reaction vessel was 6 0 mM When the cholinesterase activities of brain and ganglion toward these substrates were compared without and with inhibitors The substrate concentration was varied from 3 to 100 mM in the pS activity study and from 1 25 to 16 7 mM in the Lineweaver Burk analysis and in the study on substrate combinations Poorly soluble α naphthyl acetate was used in 0 5 mM concentration only

The selective inhibitor of specific cholinesterase (Bayliss and Todrick 1956) 1 5 bis (4 allyl dimethylammoniumphenyl) pentan 3 one diiodide (284 C 51 Wellcome Research Laboratories *England*) *was used in a concentration of* 10^{-6} *M and tetraisopropylpyrophosphoramide* (iso-OMPA Light England) was employed in a concentration of 10^{-6} M when the brain and ganglion activities were compared The concentration of 284 C 51 was 10^{-8} M in the pS activity study and in the Lineweaver Burk procedure

Enzyme assay

The cholinesterase activity was measured by automatic titration of the acid liberated by enzymatic hydrolysis with 0 005 N sodium hydroxide at pH 8 and 25 C under constant stirring and CO_2 free nitrogen flow using the Radiometer Titrigraph (Jensen Holm *et al* 1959 Holmstedt *et al* 1963) The enzyme activity was determined in a final volume of 5 0 ml The final homogenate concentration in the reaction vessel was 10 mg/ml of brain and 0 4—0 8 mg/ml of sympathetic ganglion When homogenate and enough of 0 1 M KCl had been added to make up the volume of 4 5 ml in the reaction vessel the titrigraph was started and the pH adjusted to 8 After equilibration 0 5 ml of substrate solution was added The paper speed in all experiments was 20 mm/min The sodium hydroxide consumption was recorded for 2 min Straight line recordings were obtained with the above mentioned enzyme concentrations and with all substrates used in this study Under these conditions of the present study initial velocities of enzymatic hydrolysis are recorded They were found to be proportional to the tissue concentration of the homogenates All values were corrected for spontaneous hydrolysis which was measured in each experiment

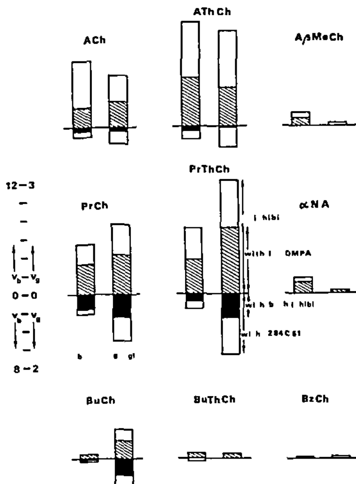


Fig 1 Enzymatic hydrolysis of different substrates by rat brain and ganglion homogenates at pH 8 with 6 0 mM substrate concentration Hydrolysis velocities of enzymatic hydrolysis of brain homogenate (v_b) and ganglion homogenate (v_g) are given in terms of ×10 ¹ μmol/ml/m n S nce 1 ml of brain homogenate contained 100 mg of fresh ti sue each number di ectly gives the activity in terms of μmol/g fre h tissue/min Since 1 ml of gangl on homogenate contained 5 mg of fresh t ssue the numbers given fo g must be mult plied by 20 to obtain the activity n similar terms Open columns upwards enzymatic activity ithout inhibitor shaded column enzymatic activity after preincubation in and in the presence of 10 ⁶ M iso-OMPA open columns downw ards enzymatic a ti ity n the presence of 10 ⁶ M 284 C 51 black columns enzymatic activity remain ng after inh bition with both 10 ⁶ M so-OMPA and 10 ⁶ M 284 C 51

When the organophosphorus inhibitor i o-OMPA was used the enzyme was preincubated in a separate closed test tube for 30 min with the inh bitor Thereafter 4 5 ml of the content of the test tube wa tran ferred into reaction ves el and 0 5 ml of substr te solution as added In the case of the competit e nhibitor 284 C 51 the inl ibitor was added into eaction essel after the acti ity ithout inh bitor v as ecord d allowing it to react for 10 m n with the enzyme before the enzymatic hydrolysis again was recorded

Protein content of the homogenates was measured by the method of Lowry *et al* (1951) with bovine serum albumin as tandard

TABLE I Cholinesterase activity of brain and ganglion homogenates at pH 8.0 with 6.0 mM substrate concentration. Activity is expressed in terms of μmoles/g protein/min

Substrate	Brain	Ganglion
Acetylcholine	119.2	376.4
Acetylthiocholine	186.5	690.3
Acetyl β metylcholine	23.5	9.1
Propionylcholine	97.4	507.6
Propionylthiocholine	119.3	878.9
α naphthyl acetate	27.7	27.3
Butyrylcholine	8.4	241.8
Butyrylthiocholine	10.1	53.3
Benzoylcholine	2.0	15.0

Results

Activity towards different substrates

The activities of brain and ganglion homogenates towards different substrates are shown in Fig. 1. The total activity obtained without inhibitors is given by the open columns upwards, the activity after preincubation in and in the presence of 10^{-6} M iso-OMPA is shown by the shaded columns, the activity remaining in the presence of 10^{-6} M 284 C 51 is given by the open columns downwards from the zero line, and the activity remaining when both 10^{-6} M iso OMPA and 10^{-6} M 284 C 51 were simultaneously used is indicated by the black columns downwards (see the arrows in the data for propionylthiocholine in Fig. 1). The scale of hydrolysis rates for brain (v_b) and ganglion (v_g) have been adjusted for convenient comparison of the relative substrate characteristics of the cholinesterase in these two tissues. Table I shows the activities obtained without inhibitors expressed in terms of μmoles of ester hydrolyzed in min per g protein.

It is evident from Fig. 1 that both brain and ganglion homogenates well hydrolyzed acetylcholine, acetylthiocholine, propionylcholine and propionylthiocholine. As compared with the activities towards these substrates, both brain and ganglion hydrolyzed less the other substrates. Acetyl β methylcholine and α naphthyl acetate were hydrolyzed at a relatively higher rate by the brain homogenates, while butyrylcholine was better split by the ganglion homogenates. Both brain and ganglion had low hydrolysis rates towards butyrylcholine and benzoylcholine.

The activity of brain homogenates towards propionylcholine and propionylthiocholine was more inhibited by 10^{-6} M 284 C 51 than the activity of ganglion homogenates towards the same substrates, and the same applied to a less extent to acetylcholine and acetylthiocholine. The activity towards butyrylcholine was unaffected by this inhibitor. On the other hand, the brain activity towards acetyl β methylcholine and α naphthyl acetate was totally abolished with 284 C 51.

In the 10^{-6} M concentration employed, iso OMPA caused considerable inhibition of the activity of brain and ganglion homogenates towards almost all substrates with relatively small differences between the substrates.

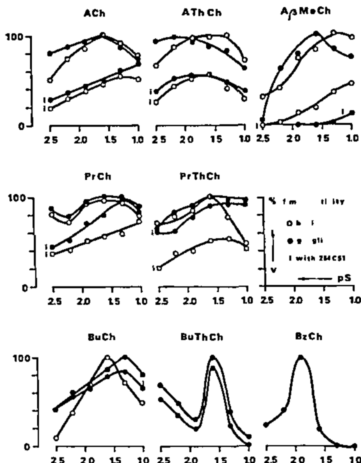


Fig 2 Activity pS curves for the enzymatic hydrolysis of different substrates by rat brain and ganglion homogenates at pH 8 pS log molar concentration of substrate v activity expressed as per cent of maximum activity for each substrate i activity in the presence of 10^{-8} M of 284 C 51 expressed as per cent of maximum activity towards the same substrate without inhibitor

When both inhibitors were used together the activity towards acetylcholine and acetylthiocholine almost completely disappeared but there was distinctly measurable activity left towards propionylcholine propionylthiocholine and butyrylcholine

Activities towards butyrylthiocholine and benzoylcholine of brain and ganglion those towards acetyl β methylcholine and α naphtyl acetate of ganglion and that towards butyrylcholine by brain were too low to permit reliable assessment of the effect of inhibitors

Inhibition by excess substrate

To investigate the effect of excess substrate on the velocity of enzymatic hydrolysis the molar substrate concentration was varied and its negative logarithm pS was

TABLE II. Michaelis Menten constants estimated from data shown in Fig. 3 and 4

Substrate	Brain	Ganglion
Acetylcholine	6.0 × 10	1.6 × 10
Acetylthiocholine	2.5 × 10	1.9 × 10
Propionylcholine	6.0 × 10	3.3 × 10
Propionylthiocholine	2.5 × 10	2.0 × 10

plotted against the activity, expressed as per cent of maximum activity for each substrate. The results are given in Fig. 2, in which is also shown the effect of 10^{-5} M 284 C 51 towards each substrate (curves marked i), again expressed as per cent of maximum activity towards the same substrate without inhibitor.

With increasing substrate concentration (decreasing pS) some inhibition was observed in the activity towards all the substrates examined when no inhibitors were used. However, the curves usually showed a decrease of activity first at very high substrate concentrations near pS 1.

On the other hand, when 284 C 51 was present the activity towards acetylcholine, acetyl-β-methylcholine, propionylcholine and propionylthiocholine showed a marked increase from pS 2.5 to pS 1.0, as can be expected of non-specific or pseudocholine esterase. Brain activities towards propionylcholine and propionylthiocholine were much more sensitive to 284 C 51 than those of ganglion towards the same substrates.

To obtain an estimate of specific or true cholinesterase activity, that is activity sensitive to 284 C 51, the activity resistant to 284 C 51 was subtracted from the total activity without inhibitor. This true cholinesterase activity towards acetylcholine, acetylthiocholine, propionylcholine or propionylthiocholine (not shown in Fig. 2) decreased markedly from pS 2.5 or 2.0 to pS 1.0. Similar tendency was less distinct with acetyl-β-methylcholine and the 284 C 51 sensitive activity of ganglion towards butyrylcholine increased rather than decreased with increasing substrate concentration.

Because of the low activities of both brain and ganglion homogenates toward butyrylcholine, butyrylthiocholine and benzoylcholine and the activity of brain homogenate towards butyrylcholine (Fig. 1), the determinations were inaccurate and care is necessary in judging the curves illustrating the pS dependence of these substrates in Fig. 2, which seem to suggest considerable inhibition with excess substrate.

Kinetics of enzymatic hydrolysis and inhibition

The interaction between substrate concentration and enzyme activity was further analyzed using the Lineweaver-Burk procedure, using the best hydrolyzed substrates acetylcholine, acetylthiocholine, propionylcholine and propionylthiocholine. In these experiments lower substrate concentrations were included and the concentrations were selected to give even spread of the 1/S values. Two concentrations of 284 C 51 were employed, 10^{-5} M and 10^{-6} M, but for the sake of clarity only the curves obtained with the 10^{-5} M inhibitor are shown in Fig. 3 and 4, the higher concentration causing such a powerful inhibition that it was not possible to fit the data in the figure.

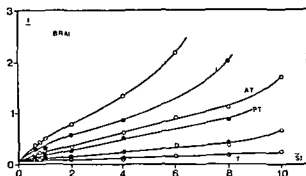


Fig 3 Enzymatic hydrolysis of acetylcholine acetylthiocholine propionylcholine and propionyl thiocholine by rat brain homogenate analyzed by the Lineweaver Burk procedure A acetylcholine AT acetylthiocholine P propionylcholine PT propionylthiocholine i corresponding curves obtained in the presence of 10 M 284 C 51 (S) 10^{-2} mM substrate concentration (when l/S is given as 10 the substrate concentration is 1 mM) v velocity of enzymatic hydrolysis 10^{-2} µmol/mg of fresh tissue/min (when l/v is given as 3 the velocity is 33 3 µmol/mg of fresh tissue/min)

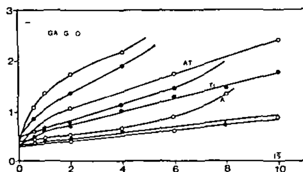


Fig 4 Enzymatic hydrolysis of acetylcholine acetylthiocholine propionylcholine and propionyl thiocholine by rat ganglion homogenate analyzed by the Lineweaver Burk procedure A acetyl choline AT acetylthiocholine P propionylcholine PT propionyl thiocholine i corresponding curves obtained in the presence of 10^{-8} M 284 C 51 S 10^{-2} mM substrate concentration (when l/S is given as 10 the substrate concentration is 1 mM) v velocity of enzymatic hydrolysis 5×10^{-2} µmol/mg of fresh tissue/min (when l/v is given as 3 the velocity is 166 5 µmol/mg of fresh tissue/min)

Except for some curving upwards at low concentrations (right) of acetylcholine and propionylcholine the values obtained without inhibitors with the four substrates and both brain (Fig 3) and ganglion (Fig 4) homogenates fell reasonably well along straight lines thus allowing the graphic estimation of the apparent Michaelis Menten constants These constants are given in Table II

Highest activities (lowest values of l/v in Fig 3 and 4) were obtained with acetylthiocholine (AT) and propionylthiocholine (PT) This applied to both brain and ganglion homogenates All the lines obtained for brain or ganglion with the 4 substrates intersected the ordinate at about the same level indicating approximately equal maximum velocities towards these substrates The curves of brain activity towards acetylcholine and propionylcholine were identical while the line illustrating the activity of ganglion homogenates towards propionylcholine was distinctly higher than that showing activity towards acetylcholine indicating lower activity

TABLE II Michaelis Menten constants estimated from data shown in Fig 3 and 4

Substrate	Brain	Ganglion
Acetylcholine	$6\ 0 \times 10$	$1\ 6 \times 10$
Acetylthiocholine	$2\ 5 \times 10$	$1\ 9 \times 10^{3}$
Propionylcholine	$6\ 0 \times 10$	$3\ 3 \times 10^{3}$
Propionylthiocholine	$2\ 5 \times 10^{3}$	$?\ 0 \times 10$

plotted against the activity expressed as per cent of maximum activity for each substrate The results are given in Fig 2 in which is also shown the effect of 10^{8} M 284 C 51 towards each substrate (curves marked i) again expressed as per cent of maximum activity towards the same substrate without inhibitor

With increasing substrate concentration (decreasing pS) some inhibition was observed in the activity towards all the substrates examined when no inhibitors were used However the curves usually showed a decrease of activity first at very high *substrate concentrations near pS 1*

On the other hand when 284 C 51 was present the activity towards acetylcholine acetyl β methylcholine propionylcholine and propionylthiocholine showed a marked increase from pS 2 5 to pS 1 0 as can be expected of non specific or pseudocholine esterase Brain activities towards propionylcholine and propionylthiocholine were much more sensitive to 284 C 51 than those of ganglion towards the same ubstrates

To obtain an estimate of specific or true cholinesterase activity that is activity sensitive to 284 C 51 the activity resistant to 284 C 51 was subtracted from the total activity without inhibitor This true cholinesterase activity towards acetyl choline acetylthiocholine propionylcholine or propionylthiocholine (not shown in Fig 2) decreased markedly from pS 2 5 or 2 0 to pS 1 0 Similar tendency was less distinct with acetyl β methylcholine and the 284 C 51 sensitive activity of ganglion towards butyrylcholine increased rather than decreased with increasing substrate concentration

Because of the low activities of both brain and ganglion homogenates towards butyrylcholine butyrylthiocholine and benzoylcholine and the activity of brain homogenate towards butyrylcholine (Fig 1) the determinations were inaccurate *and care is necessary in judging the curves illustrating the pS dependence of these* substrates in Fig 2 which seem to suggest considerable inhibition with excess sub strate

Kinetics of enzymatic hydrolysis and inhibition

The interaction between substrate concentration and enzyme activity was further analyzed using the Lineweaver Burk procedure using the best hydrolyzed substrates acetylcholine acetylthiocholine propionylcholine and propionylthiocholine In these experiments lower substrate concentrations were included and the concentrations were selected to give even spread of the 1/S values Two concentrations of 284 C 51 were employed 10^{8} M and 10^{6} M but for the sake of clarity only the curves obtained with the 10^{8} M inhibitor are shown in Fig 3 and 4 the higher concentra tion causing such a powerful inhibition that it was not possible to fit the data in the figure

Hydrolysis of mixtures of substrates

The rates of hydrolysis of acetylcholine propionylcholine and propionylthiocholine by brain homogenates were further examined at different substrate concentrations when 2 substrates were present at the same time The results are shown in Fig 5

Fig 5 A illustrates the interaction of acetylcholine and propionylcholine The first column in each set of 3 columns shows the activity towards acetylcholine at the concentration indicated the second column shows the activity towards propionyl choline at the same molar concentration and the third column shows the activity observed in the presence of both sub trates at the same concentration Thus the total molar substrate concentration in the last of the 3 columns was always twice that in the first 2 columns The third column must therefore be compared also with the activity obtained with acetylcholine or propionylcholine alone at twice the concentration In the first triplet of Fig 5 A showing activity at 1 25 mM substrate concentration the third column is higher than either of the 2 first columns in the first triplet but lower than either of the first 2 columns of the second triplet obtained at twice the substrate concentration In the third triplet obtained with 5 0 mM concentration of each substrate the activity obtained with the mixture of the 2 substrates is about the same order of magnitude as those obtained with either substrate alone This indicates saturation of the enzyme with substrate

Essentially similar results were also obtained with the other substrate combinations examined (Fig 5 B C D and E) At the lowest substrate concentration 1 25 mM an increase of activity was obtained when both substrates were present together Such an increase was small or absent at the concentration of 2 5 mM and it was always absent at the concentration of 5 0 mM At 16 7 mM substrate concentration inhibition by excess substrate was observed that is the combination of acetylthiocholine and propionylcholine (Fig 5 B) or acetylthiocholine and acetylcholine (Fig 5 C) resulted in an activity smaller than that obtained with any of these substrates alone None of the pairs of substrates showed summation of activity such as has been shown to be the case when 2 enzymes act separately on each substrate Augustinsson 1948)

Discussion

The relatively high specificity of acetyl β methylcholine to specific cholinesterase and similar *specificity of butyrylcholine to non specific cholinesterase earlier reported in* many studies (see reviews by Augustinsson 1948 1963 and Whittaker 1951) was confirmed in the present study and it was also ascertained that specific cholinesterase dominates in brain (Ord and Thompson 1952 Myers 1953 Bergmann and Segal 1955 Bayliss and Todrick 1956) while more non specific cholinesterase is present in the sympathetic ganglion (Glick 1938 Sawyer and Hollinshead 1945 Klingman *et al* 1968 Harkonen and Penttila 1971)

However it was shown in the pre ent study that not only acetylcholine and acetyl thiocholine but also propionylcholine and propionylthiocholine are readily hydrolyzed by both specific and non specific cholinesterase 10^{-6} M 284 C 51 which totally

inhibited the activity towards acetyl β methylcholine but had hardly any effect on the activity towards butyrylcholine inhibited only a part of the activity towards these 4 substrates and a part of the remaining activity was sensitive to iso OMPA That acetylcholine and acetylthiocholine are hydrolyzed by both specific and non specific cholinesterase has long been known (Whittaker 1951, Augustinsson 1963 Eränkö 1972) Cholinesterase activity towards propionylcholine has earlier been observed in the horse serum (Glick 1938) sera of several other mammalian and avian species (Myers 1953) the rat heart (Ord and Thompson 1951) as well as brain of the rat (Ord and Thompson 1952 Myers 1953 Eränkö 1972) man (Ord and Thompson 1952) and the chicken (Myers 1953) This activity has been considered to be due to a propionylcholinesterase an enzyme classified to the same group of non specific or pseudocholinesterases as butyrylcholinesterase (Ord and Thompson 1952 Augustinsson 1963)

In the present study it was shown that propionylcholine is indeed split by non specific cholinesterase However the data obtained with the selective inhibitors 284 C 51 and iso OMPA (see also Eränkö 1972) indicate that propionylcholine also serves as a substrate for specific cholinesterase

This is further supported by kinetic observations Inhibition by excess substrate has been considered to be a typical characteristic of specific cholinesterase (Whittaker 1951 Bergmann and Segal 1955 Glick 1938 Mendel and Rudney 1944 Sawyer and Hollinshead 1945 Augustinsson 1948 Myers 1953 Cohen and Oosterbaan 1963) Of the total cholinesterase activity towards propionylcholine and propionylthiocholine the part which was ascribed to specific cholinesterase on the basis of its sensitivity towards 284 C 51 showed in the present study a pronounced decrease when the substrate concentration was increased like the specific activity towards acetylcholine and acetylthiocholine The observations of the present paper are in this respect similar to those made by Myers (1953) on the activity of rat and chicken brain homogenates towards acetylcholine and propionylcholine in the presence of 10^{-8} M di isopropylfluorophosphonate which led him to conclude that also specific cholinesterase can hydrolyze propionylcholine

The effect of substrate concentration on the enzyme activity was examined in the present study which the Lineweaver Burk procedure using tissue homogenates instead of purified enzyme preparations Although some care is therefore necessary in interpreting the results it is of considerable interest that the apparent Michaelis Menten constant for brain activity towards acetylcholine was equal to that of propionylcholine and on the other hand that the brain constants towards acetylthiocholine and propionylthiocholine were also equal Since the cholinesterase activity of brain homogenates was mainly due to specific cholinesterase this can be taken as evidence for propionylcholine and propionylthiocholine serving as substrates for specific cholinesterase The approximately similar Michaelis Menten constants of ganglion activity towards acetylcholine acetylthiocholine propionylcholine and propionylthiocholine are also of interest even *if all these substrates are hydrolyzed both by specific* and by non specific cholinesterase present in the ganglion homogenates

Lineweaver Burk data obtained with inhibitors are difficult to interprete because the inhibition by 284 C 51 was much less at high substrate concentrations than could be expected if the inhibition had been purely competitive This suggests that at high substrate concentrations an enzyme mechanism sets in which is not competitively inhibited by 284 C 51 although at lower substrate concentrations this inhibitor presumably acts upon the same active centre that combines with the substrate This interpretation is the same as that given by Augustinsson (1948) for the non linear inhibition by choline which he observed in the activity of cow erythrocytes towards acetylsalicylcholine In the present case the participation of both specific and non specific cholinesterase in the enzymatic hydrolysis of the substrates can also be expected to cause resistance to inhibitor at high substrate concentration because only the former is sensitive to 284 C 51 Indeed the activity pS curves of ganglion homogenates which contain more non specific cholinesterase than the brain homo genates showed much more curving than the latter

Experiments with mixtures of substrates always showed a level of activity with two different substrates which was approximately equal to that obtained with twice the concentration of either of these two substrates Since no additive effect was observed it seems clear that all the 4 choline esters tested in this experiment that is acetyl choline acetylthiocholine propionylcholine and propionylthiocholine were hydrolyzed by the same enzyme(s) in the brain homogenate mainly by specific cholin esterase For further considerations on hydrolysis of mixtures of substrates the reader is referred to Augustinsson s fundamental study (1948)

Thus different kinds of evidence were obtained in the present study for the view that propionylcholine serves as a substrate for specific cholinesterase This has been previously proposed by Myers (1953) on the basis of activity pS characteristics of brain cholinesterases and the data now obtained using the highly selective inhibitor of specific cholinesterase 284 C 51 lends especially strong support to this view

In the present study propionylthiocholine has been for the first time shown to serve as a substrate for both specific and non specific cholinesterase in much the same way as propionylcholine This is of considerable interest because propionyl thiocholine being a thioester can be used as a histochemical substrate Only one report by Atherton (1963) is as yet available dealing with the histochemical use of this substrate He found that the localization of the reaction obtained with propionyl thiocholine in the chick brain was often similar to but sometimes different from that obtained with acetylthiocholine as a substrate Since Atherton s study (1963) was carried out without the use of selective inhibitors his observations are difficult to interprete Therefore further histochemical work with propionylthiocholine would be of obvious interest

Since several choline esters beyond any doubt serve as substrates for both specific and non specific cholinesterases (see above) and since moreover some tissues such as the chicken brain hydrolyze propionylcholine at a higher rate than acetylcholine terms such as acetylcholinesterase propionylcholinesterase and butyrylcholinesterase have been avoided in the present study To quote Myers (1953) it does seem justifi

able and useful to retain the distinction between the 2 main groups of eserine sensitive cholinesterases in vertebrate physiology. Since non specific cholinesterase is localized in sympathetic ganglion mainly in the glial elements (Koelle 1951, 1963, Harkönen 1964), which may significantly take part in the hydrolysis of transmitter substances, this term appears preferable to the term pseudocholinesterase which implicates that the cholinesterase activity not being true is without functional significance.

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The Effect of Stimulation of the Cervical Sympathetic Chain on Retinal Oxygen Tension and on Uveal, Retinal and Cerebral Blood Flow in Cats

By

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Abstract

ALM A and A BILL *The effect of stimulation of the cervical sympathetic chain on retinal oxygen tension and on uveal retinal and cerebral blood flow in cats* Acta physiol scand 1973 *88* 84—94

The effects of stimulation of the cervical sympathetic chain on blood flow through the retina the different parts of the uvea different parts of the brain and some other tissues were studied in cats 5 Hz reduced the oxygen tension in the vitreous body close to the retina in 5 expts of 6 15 μm labelled microspheres were used for quantitative determinations of blood flow Unilateral stimulation at 10 Hz was performed for 1 min in 9 normocapnic and in 4 hypercapnic cats and for 15—30 min in 5 normocapnic cats The results suggest that blood flow through the innermost layers of the retina is influenced by sympathetic stimulation The vascular resistance within the uvea was much increased in all three groups with no indications for autoregulatory escape The effect was most pronounced in the ciliary processes and least in the ciliary muscle Autoregulatory escape was seen in the parotid gland the tongue and the masseter muscle where 1 min stimulation caused mean reductions in blood flow by 82—99 compared to 6—56 after 15—30 min stimulation Cerebral blood flow showed no statistically significant reductions in either group indicating a quantitatively minor role for the sympathetics in regulation of total cerebral blood flow in cats

Stimulation of the cervical sympathetic chain is known to reduce total uveal blood flow in cats (Bill 1962) while the effect on the blood flow through the retina is unknown It has been shown for both cats and primates that the retinal vessels unlike those of the uvea lack adrenergic innervation (Laties and Jacobwitz 1966 Ehinger and Falck 1969) and thus the sympathetics can exert no direct influence on the retinal arterioles Still there are good reasons to suspect that stimulation of the cervical sympathetic chain may change the blood flow through the retina The ophthalmic artery and its narrow branches to the retina are innervated up to lamina cribrosa in the optic nerve head Constriction of the arteries outside the eye will reduce the perfusion pressure for blood flow through the retina which will tend to decrease retinal blood flow However a moderate reduction might be compensated by retinal

vasodilatation and retinal blood flow might even increase to compensate reduced choroidal blood flow Such an effect is seen when ocular perfusion pressure is reduced by increments in eye pressure (Alm and Bill 1972 b)

The purpose of the experiments reported here was to study the influence of the cervical sympathetics on retinal blood flow and oxygen supply Two previously de scribed methods were used 1 continuous determination of the oxygen tension in the vitreous body close to the retina P_{vrO} (Alm and Bill 1972 a) and 2 the labelled microsphere method (Alm and Bill 1972 b) With the latter method it was possible to make simultaneous determinations of blood flow through various parts of the eye and the brain through the tongue the parotid gland and the masseter muscle

Methods

Adult cats of both sexes weighing between 2 0 and 3 5 kg were used Anesthesia was induced with chloroform and maintained by i v injections of chloralose 50—70 mg/kg b w and if necessary urethane 100—300 mg/kg b w The femoral artery and ve n on one side were cannulated the vein for infusions and the artery for measurements of the air-damped mean arterial blood pressure (MAP) with a pressure transducer (EMT 490 A Elema Schonander Solna Sweden) All animals were tracheotomized and artificially ventilated by a Palmer pump Arterial P_O P_{CO} and pH were determ ned with a Beckman Physiolog cal Gas Analyzer Model 160 (see Alm and Bill 1970) Heparin 2500 I U was given i v to prevent clott ng The animals were placed on a heating pad and the body temperature determined with a rectal thermometer was maintained at 36 5—39 5 C

Measurements of the oxygen tension close to the retina P_{vrO}

The method has been described in detail previou ly (Alm and Bill 1972 a) In 6 animals P_{vrO} was continuously determ ned by means of a membrane covered oxygen electrode (Beckman oxygen micro electrode) inserted into the eye through the scle a and placed with the tip in the vitreous body within 2 mm from the retina The anterior chamber was cannulated with a steel needle for stabilizat on of the intraocular pressure (IOP) by means of an external reservoir and for measure ments of the actual IOP with a p e sure transducer (EMT 490 A) IOP MAP and P_{vrO} were recorded on a mult point recorder (Ph l ps PR 3212 A/00)

Both cervical sympathet c chains were cut and the homolateral d stal end on one side was sti mulated with a bipolar plat num electrode nterelectrode distance 1 mm connected to a stimu l tor AEL model 111 (Amer can Electronic Laborator es) The electrode was placed below the superior cerv cal sympathetic gangl on and the area was flooded with mineral oil to prevent drying of the nerve Square wave pulses of 1 or 10 m duration were used at a stepw se increased frequency The st mul tion intensity was adjusted to give maximal pupillary response usually 6—8 V Dur ng p olonged st mulat ons it was so net mes necessary to move the electrode sl ghtly to mainta n ade quate pup llary response Changes in st mulation frequency were made at steady levels of P_{vrO} In 4 e periments st mulation was also perfo med at 10 or 20 Hz after i adm n st t on of 40 mg phentolamine (Regit n[R] Ciba)

The labell d mic phere meth d

The method as used by us has been described n detail previously (Alm and B ll 1972 b) As a rule 0 5—1 0 ml of a 1 su pension of 15 μm m cropsh es n aline was used The spheres were labelled with Sr or Ce nd the specif c activity was 5—10 mC /g The m crospheres (3 M Company St Paul M nne ota) we e njected into the left ent cle of the heart The injection took about 10 s and during that t me nd the follow ng 50 s blood was collected f om a cannulated f moral artery to obtain a r ference flow At th end of the 60 s pe od th nimal as killed by i v KCl and d sected The r d oacti ty was determined by g mm pectrometry and the blood flow through each ti ue mple wa calculated acco d ng to the formula

$$\text{Blood flow} = \frac{\text{activity n t ssue} \times \text{refe ence blood flow}}{\text{act v ty n reference blood}}$$

where blood flow expressed n mg/m n and activity in cpm

As a rul doubl or quadruple s mples w t k n f om a ou reg ons of the bra n and from the tongue the p otid glands and the m t muscles Both y were enucleated and diss ct

as previously described (Alm and Bill 1972 b) so that separate samples of the iris the ciliary body the retina and the choroid were obtained In this series attempts were made to determine the flow through the ciliary processes and the ciliary muscle separately To facilitate dissection of the ciliary body it was fixed in 4 glutaraldehyde for at least 48 h and then dehydrated in graded ethanol (30 50 70 95 and 100) Before being allowed to air dry the specimens were dissected under an operation microscope to yield pieces representing mainly ciliary processes and ciliary muscle respectively Ciliary muscle was obtained by cutting pieces from the scleral surface of the fixed ciliary body as a rule the samples did not include the subepithelial part of the ciliary muscle The activity of the total ciliary body was determined before dehydration absorption of the gamma radiation in the sample was negligible

Three types of experiments were performed with the microsphere method The stimulation frequency for all animals was 10 Hz the stimulus duration was 1 or 10 ms and the intensity 6–8 V In one group stimulation was performed for 1 min before flow determination in 9 normocapnic animals in another group it was maintained for 15–30 min in 5 normocapnic animals and in a third group stimulation was maintained for 1 min in 4 hypercapnic animals ventilated with a mixture of room air and carbon dioxide

Statistics

In the following mean and S E are given Student s t test based on paired data was used to analyse the effects of stimulation Group comparison was made with Student s t test if an F test did not show statistically significant difference in variance between the two groups ($p > 0.05$) Else a nonparametric test the Mann Whitney U test (Siegel 1956) was used

Results

Starting data

In the animals artificially ventilated on room air the following data were obtained, MAP 182±7 cm H_2O (n = 20) arterial P_{O_2} and P_{CO} 98±2 mm Hg (n = 20) and 31±1 mm Hg (n = 20) respectively In the animals where P_{VRO} was determined at stabilized IOP the following additional parameters were recorded IOP 32±2 cm H_2O (n = 6) and P_{VRO} 29±4 mm Hg (n = 6) For animals ventilated on a mixture of room air and carbon dioxide the following data were obtained MAP 180±18 cm H_2O (n = 4) arterial P_O P_{CO_2} and pH 100±2 mm Hg (n = 4) 70±1 mm Hg (n = 4) and 7 18±0 03 units (n = 4) respectively

The oxygen tension close to the retina P_{VRO_2}

Fig 1 shows how P_{VRO} changed in 6 animals subjected to stimulation of the cervical symphathetic chain In 5 expts there was a clear reduction in P_{VRO_2} already at a stimulation frequency of 2–5 Hz and this reduction could not be augmented by higher frequencies The mean reduction for all animals at 5 Hz was 20±7 % ($p < 0.05$) In 3 of the expts 40 mg phentolamine was administered i v while the stimulation was continued at 20 Hz this resulted in a return of the P_{VRO_2} to the prestimulation level (not shown in the fig) In these and one further experiment renewed stimulation at 10 Hz after 40 mg phentolamine i v caused no change in P_{VRO_2}

Blood flow determined with microspheres

Table I presents the flow values for the sympathectomized control side in normocapnic and hypercapnic animals Weights were not determined for the retina or the choroid and for these as for the rest of the uvea flow is presented as total flow in the whole tissue For the ciliary body and its parts dry weight was determined and used

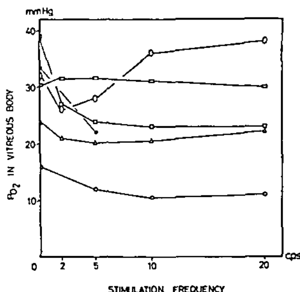


Fig. 1. The oxygen tension in the vitreous body close to the retina, P_{VTO}, was continuously determined in 6 cats while the distal end of the divided ipsilateral cervical sympathetic chain was stimulated at stepwise increased frequencies. Changes in stimulation frequency were made at steady levels of P_{VTO}.

TABLE I. Blood flow through sympathectomized side. Mean ± S.E. (n) = number of animals

Tissue	Normocapnic animals		Hypercapnic animals	
	Blood flow in whole tissue (mg/min)	Blood flow (g/min per 100 g tissue)	Blood flow in whole tissue (mg/min)	Blood flow (g/min per 100 g tissue)
Retina	25 ± 4 (14)		55 ± 5 (4)	
Iris	27 ± 5 (14)	43 ± 8 (14)	245 ± 26 (4)	367 ± 47 (4)
Ciliary body	333 ± 38 (14)	280 ± 28 (14)	803 ± 74 (4)	632 ± 62 (4)
Choroid	1271 ± 172 (14)		1674 ± 398 (4)	
Ciliary processes		556 ± 58 (14)		843 ± 70 (4)
Ciliary muscle		96 ± 10 (14)		287 ± 90 (4)
Parotid gland		16 ± 3 (14)		9 ± 3 (4)
Tongue		29 ± 3 (14)		11 ± 3 (3)
Masseter muscle		15 ± 3 (14)		7 ± 3 (4)
Grey matter				
Frontal lobe		46 ± 4 (11)		388 ± 20 (4)
Temporal lobe		34 ± 3 (11)		281 ± 33 (4)
Occipital lobe		52 ± 5 (11)		364 ± 44 (4)
White matter				
Frontal lobe		45 ± 4 (11)		19[illegible] ± 10 (4)
Temporal lobe		29 ± 3 (11)		168 ± 2 (4)
Occipital lobe		54 ± 5 (11)		154 ± 13 (4)
Corpus callosum		20 ± 4 (11)		123 ± 20 (3)
Choroid plexus		301 ± 32 (11)		143 ± 38 (4)

TABLE II Percent reduction of blood flow on stimulated side Mean ± S E (n) = number of animals

Tissue	1 stim Normocapnia		15–30 sim Normocapnia		1 stim Hypercapnia	
Retina	−44 ± 39	(9)	− 8 ± 24	(5)	6 ± 21	(4)
Iris	50 ± 8	(9)***	28 ± 11	(5)*	76 ± 9	(4)**
Ciliary body	66 ± 4	(9)***	67 ± 6	(5)***	63 ± 13	(4)*
Choroid	61 ± 3	(9)***	67 ± 7	(5)***	42 ± 12	(4)*
Ciliary processes	77 ± 5	(9)***	77 ± 8	(5)***	76 ± 5	(4)***
Ciliary muscle	16 ± 14	(9)	28 ± 13	(5)*	46 ± 18	(4)*
Parotid gland	99 ± 1	(9)***	34 ± 15	(5)	77 ± 21	(4)*
Tongue	82 ± 4	(9)***	56 ± 11	(5)**	39 ± 37	(3)
Masseter muscle	96 ± 3	(9)***	6 ± 65	(5)	55 ± 36	(4)
Grey matter						
Frontal lobe	20 ± 9	(6)	−11 ± 17	(5)	8 ± 12	(4)
Temporal lobe	9 ± 10	(6)	16 ± 13	(5)	2 ± 13	(4)
Occipital lobe	− 8 ± 5	(6)	−15 ± 17	(5)	6 ± 13	(4)
White matter						
Frontal lobe	12 ± 3	(6)*	−20 ± 12	(5)	18 ± 9	(4)
Temporal lobe	8 ± 7	(6)	6 ± 31	(5)	15 ± 10	(4)
Occipital lobe	4 ± 5	(6)	10 ± 3	(5)	9 ± 22	(4)
Choroid plexus	8 ± 6	(5)	6 ± 4	(5)	−18 ± 39	(4)

Asterisks indicate significance level based on Student's t test paired data * $p < 0.05$ ** $p < 0.01$ *** $p < 0.001$ Percent reduction = 100 (blood flow through control side minus blood flow through stimulated side) (blood flow through control side)

to estimate wet weight on the assumption that the ratio (dry weight)/(wet weight) is 0.20 (see Alm and Bill 1972 b) The flow through the ciliary processes was much higher than that through the ciliary muscle The difference in normocapnic animals based on paired data 461 ± 54 g/min per 100 g tissue (n = 14) is highly significant ($p < 0.001$) In the brain of normocapnic animals the differences between grey and white matter were small and insignificant while there were differences in flow between the various regions Thus the flow through white matter for each lobe differed significantly ($p < 0.01$) from that through the other lobes while for grey matter flow through the temporal lobe was lower than that through the occipital lobe ($p < 0.001$) and that through the frontal lobe ($p < 0.05$) The statistical analyses are based on paired data

Blood flow rates in cerebral and ocular tissues were generally higher in hypercapnic animals than in normocapnic animals while for the parotid gland the tongue the masseter muscle and the choroid plexus they tended to be lower In hypercapnic cats there seem to be a difference in flow between grey and white matter The flow through grey matter is higher than that through white matter in the frontal lobe ($p < 0.005$) and in the temporal and the occipital lobes ($p < 0.05$) The p values were calculated using paired data

Table II presents the effects of stimulation of the cervical sympathetic chain in the three groups 1 min stimulation caused marked reductions of blood flow through the various parts of the uvea the parotid gland the tongue and the masseter muscle

in normocapnic animals while there were no statistically significant changes in flow through the retina the ciliary muscle or the cerebral tissues (with the possible exception of frontal white matter) In the few hypercapnic animals the results were similar although the average reduction in flow in extraocular tissues was less pronounced Prolonged stimulation between 15 and 30 min resulted in reductions of blood flow through most ocular tissues similar to those caused by 1 min stimulation The iris was a possible exception The effect after 15—30 min was less pronounced than that after 1 min for the tongue and the masseter muscle ($p < 0.05$) and for the parotid gland ($p < 0.01$, Mann Whitney U test)

Discussion

The effect of sympathetic stimulation on the oxygen tension close to the retina P_{VrO_2}

Stimulation of the cervical sympathetic chain reduced P_{VrO} and thus the mean tissue P_{O_2} in the near by retina must have changed in the same direction The effect was due to stimulation of adrenergic alpha receptors since alpha adrenergic blockade with phentolamine abolished it Since no reduction in P_{VrO} was caused by a reduced choroidal blood flow in experiments where the IOP was raised (Alm and Bill 1972 a) and since arterial P_{O_2} was not changed the fall in P_{VrO} must have been caused by an increase in tissue O_2 consumption or by a reduction in retinal blood flow The adrenergic neurons found in the retina have no connections with the cervical sympathetic chain (Ehinger and Falck 1969) and the release of noradrenaline in the choroid can not explain the effect close i a injections of noradrenaline do not cange the P_{VrO} (Alm 1973) Thus the effect must be due to stimulation of adrenergic alpha receptors outside the retina Then the most likely explanation for the reduction in P_{VrO_2} seen in this study is a reduction in blood flow through the innermost layers of the retina due to an intense constriction of the vessels outside the eye

Blood flow through tissues on the control side determined with microspheres

The control eyes in this series differ in two respects from those presented previously (Alm and Bill 1972 b) where the cervical sympathetic chain was left intact and where the anterior chamber was cannulated with a steel needle In the previous study the mean flow values for the retina the iris the ciliary body the choroid and the total uvea were 15 60 262 734 and 1070 mg/min respectively A comparison between these values and those of table 1 shows that in the present study blood flow through the iris is lower while that through the other tissues is higher than the earlier reported values The differences are statistically significant for the iris and the choroid ($p < 0.01$ Student s t test group comparison) Loss of sympathetic tone may explain the difference in blood flow through the choroid since dividing the cervical sympathetic chain in cats tends to increase total uveal blood flow by 10—20 % (Bill 1962) The higher blood flow through the iris in the previous report may be due to the small trauma to the eye caused by cannulation of the anterior chamber

Blood flow per 100 g tissue through the ciliary processes is about 5 times that through the ciliary muscle a difference much larger than that seen in monkeys with intact sympathetic innervation (Alm and Bill 1973) Since stimulation of the cervical sympathetic chain had marked effects on blood flow through the processes compared to that through the muscle normal sympathetic tone may at least partly reduce the difference in flow between the two regions However, there may also be a species difference between cats and monkeys

In the brain the ratio (flow through grey matter)/(flow through white matter) was about 1 The isotope clearance method suggests a ratio for flow between the two compartments of about 5 (Häggendal Nilsson and Norbäck 1965) in dogs, a result supported by determinations in cats with i v injections of antipyrine ^{14}C (Reivich *et al* 1969) The reason for the difference between the results obtained with micro spheres and with diffusable isotopes is not clear However axial streaming of micro spheres tends to underestimate the ratio since the spheres have a tendency to follow the main vessels Many arterioles supplying white matter give off small side branches when they pass through grey matter Thus for these vessels blood to white matter can be expected to have a higher concentration of spheres than blood to grey matter The ^{85}Kr method on the other hand is no ideal reference method for studies on the ratio (flow through grey matter)/(flow through white matter) since vessels supplying white matter first have to pass through grey matter and ^{85}Kr passes through the walls of small veins and arterioles (Sejrsen 1970) Also the antipurine method may give somewhat erroneous results since it too implies that blood tissue exchange is restricted to the capillaries Differences in anesthesia may also have contributed to the discrepancy between the results In the present study the ratio was about 2 is hyper capnic cats The difference between hypercapnic and normocapnic cats in probably due to artefacts of the microsphere method since the increases in blood flow rates in grey and white matter determined with the isotope clearance method were of the same order in dogs with varying arterial P_{CO} (Haggendal Nilsson and Norback 1965)

Effect of 1 min stimulation of the cervical sympathetic chain in normocapnic animals

Retraction of eve lids and nictitating membrane and dilatation of the pupil were used for determining adequate stimulation intensity The effect on the blood flow through the tongue the masseter muscle and the parotid gland confirmed that the stimulation was adequate In all these tissues there was a marked reduction in blood flow in the parotid gland flow was almost stopped on the stimulated side in all experiments

Retina There was no statistically significant difference in flow between the two retinas but the variance for the difference control eye minus experimental eye was great, much greater than the variance for the corresponding difference in previous cat experiments with manipulation of the perfusion pressure (Alm and Bill 1972 b) The difference in variance was significant ($p < 0.01$ F test)

Since the amounts of microspheres injected were similar the difference in variance suggests that sympathetic stimulation had an effect on retinal blood flow but that

the effect was variable The measurements of the oxygen tension close to the retina indicated that sympathetic stimulation reduces blood flow in the innermost layers of the retina The result obtained with the microspheres was an increase in total retinal blood flow but the 95 % confidence interval includes negative values for the increase Possibly due to the limited amount of data available there is thus no certain discrepancy between the results with the two methods

Uvea Stimulation of the cervical sympahtetic chain at 10 Hz results in almost maximal reduction in total uveal blood flow in both cats and rabbits In both species uveal vascular resistance including resistance in the extraocular parts of the arteries supplying the eye increases by about 130 % (Bill 1962) Using the ascorbic acid clearance method Langham and Rosenthal (1966) found that sympathetic stimulation caused a 50 % reduction in blood flow through the ciliary processes in rabbits Cole and Rumble (1970) using implanted thermocouples showed a reduction in blood flow through the iris when the cervical sympathetic chain was stimulated In contrast to these findings and those of the present experiments Best Masket and Rabinovitz (1972) reported that stimulation of the cervical sympathetic chain has no effect on the vascular resistance in the uvea in cats and rabbits They suggested that effects observed by other investigators were due to changes in pressure in the arteries supplying the eye In rabbits stimulation of the intact cervical sympathetic chain reduced femoral and ciliary arterial pressures and uveal blood flow In cats flow and pressures increased However determinations of the effect of sympathetic stimulation on the blood pressure in the ciliary arteries in rabbits indicate that although some of the increase in vascular resistance takes place proximal to the ciliary arteries most of it occurs in smaller blood vessels the pressure in the ciliary artieries rises to the same extent as that in the femoral arteries (Bill 1963) The effect on the retinal blood flow in the present study suggests that a not negligible part of the increase in vascular resistance is extraocular Nonetheless the possibility that the vascular resistance within the uvea was unchanged is highly improbable for the following reasons 1) if all increase in vascular resistance occurred in the ciliary artery which is the main arterial supply in cats (Henkind 1966) and in larger arteries the effect on the eye would have been that of a reduced perfusion pressure Then one should not expect the uniformity of reductions in blood flow through the various parts of the uvea that was found in the present study blood flow through the anterior uvea is efficiently autoregulated in contrast to that through the choroid In fact a mean reduction in choroidal blood flow in cats by 58 % through increments in the IOP left the blood flow through the anterior uvea almost unchanged (Alm and Bill 1972 b) 2) if all increase in vascular resistance occurred in the long posterior ciliary arteries which are the continuation of the ciliary artery supplying the anterior uvea and in larger arteries one should expect similar effects on blood flow through the ciliary processes and through the ciliary muscle since these tissues are supplied by the same arteries and react in similar ways to reductions in the perfusion pressure (Alm and Bill 1973) The present result a reduction in blood flow that is much more pronounced in the ciliary processes than in the ciliary muscle indicates a difference between the effects of sympathetic stimu

lation on the vascular resistance within the two tissues This is in accordance with the distribution of adrenergic nerves, the vessels of the ciliary processes have a rich adrenergic nerve supply while it is doubtful if those of the deeper parts of the ciliary muscle are supplied with adrenergic nerves (Ehinger 1966) Thus it seems likely that the changes in uveal blood flow that are caused by stimulation of the cervical sympathetic chain are due mainly to increments in the vascular resistance in the different parts of the uvea It is not clear why Best Masket and Rabinovitz (1972) failed to uncover changes in the vascular resistance in the uvea Interference with the vascular supply to the eye in their experiments or differences in stimulation techniques are possible explanations

In the group of 9 normocapnic animals with 1 min stimulation of the cervical sympathetic chain the reduction in blood flow through the ciliary muscle was not statistically significant In the group with stimulation for 30 min and in that with hypercapnia the reductions were probably significant The effects of stimulation on blood flow rate in the whole ciliary body in the three groups were very similar Then it seems justifiable to treat the three groups as a statistically homogenous material Under such conditions the reduction in blood flow through the ciliary muscle was 26 ± 9 % (n = 18) (p = < 0 01) Thus we may conclude that there is a true small reduction in blood flow through the ciliary muscle when the cervical sympathetic chain is stimulated

Brain The effect of the sympathetic nervous system on cerebral blood flow is still controversial Although the adrenergic innervation is less dense than in most vascular beds there is no doubt that at least pial vessels are innervated even vessels as small as 15—20 μm (see Nelson and Rennels 1970) Constriction of vessels down to 20 μm should have a marked effect on the vascular resistance Many investigators using different methods have failed to show any clear cut effects while others using the same methods have found indications of a more or less pronounced cerebral vasoconstriction (for a recent review see Purves 1972) Recently James Millar and Purves (1969) using the isotope clearance method found a 30 % reduction in blood flow through the cerebral cortex of the adult baboon as a result of stimulation of the cervical sympathetic chain In the present study there were no significant reductions in flow in the regions studied The vessels of the choroid plexus like the penetrating vessels of the hemispheres lack adrenergic innervation in the cat (Angelakos Irvin and King 1970)

Effect of 15—30 min stimulation of the cervical sympathetic chain

It is known from other organs that prolonged stimulation of the sympathetic may result in a reduction of the response an autoregulatory escape This is seen mainly in the intestine but also in the kidney and the liver but not in the skeletal muscle (see Mellander and Johansson 1968) In the present study autoregulatory escape was seen in the parotid gland and possibly in the tongue and the masseter muscle but not in the uvea In the uvea the degree of reduction of blood flow was the same after 15—30 min stimulation as after 1 min Thus we may assume that the escape of

the parotid gland is a true escape and not due to a failure to maintain adequate stimulation for such long time

Effect of 1 min stimulation of the cervical sympathetic chain in hypercapnic animals

James Millar and Purves (1969) found a 30 % reduction in blood flow through the cerebral cortex in normocapnic baboons as the result of stimulation of the cervical sympathetic chain and a still more pronounced effect in hypercapnic animals This led them to suggest that the failure of some investigators to observe similar effects in cats was due to the normally low arterial P_{CO} in cats, 25—30 mm Hg compared to 35—40 mm Hg in the baboon To investigate whether the low arterial P_{CO} had consequences for the reaction of retinal and cerebral blood flow 1 min stimulation was performed in 4 cats ventilated on a mixture of room air and carbon dioxide The arterial P_{CO_2} was 69—73 mm Hg In these animals the responses seem to be less pronounced and less constant for the parotid gland the tongue and the masseter muscle than in normocapnic animals No obvious differences were seen for either the ocular tissues or the cerebral tissues Although the number of experiments is small a clear cut reduction in cerebral blood flow of the order of 30 % should have been noted Measurement of the pressure drop in the arteries have shown that only about 50 % of the total vascular resistance in the cerebral cortex is situated between the aorta and pial vessels down to 25 μm (Shapiro *et al* 1971) This is about as far as the sympathetic nerves innervate the cerebral vessels (Nelson and Rennels 1970) A not negligible part of the vascular resistance thus lies out of reach of the sympathetic nervous system It is possible that sympathetic stimulation causes constriction of the pial vessels which is concealed by a dilatation of smaller vessels through autoregulatory mechanisms The fact that the blood flow remained constant during the stimulation even in hypercapnic animals indicates that such compensation is sufficient even in a situation with very marked cerebral vasodilatation This result suggests that sympathetic effects on cerebral blood flow in cats are very small indeed

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The Effects of Vasoconstrictor Fibre Stimulation on Consecutive Vascular Sections of Cat Small Intestine during Hemorrhagic Hypotension

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Abstract

HAGLUND U and O LUNDGREN *The effects of vasoconstrictor fibre stimulation on consecutive vascular sections of cat small intestine during hemorrhagic hypotension* Acta physiol scand 1973 *88* 95—108

The effects of the regional sympathetic vasoconstrictor fibres on the consecutive vascular sections of the small intestine of the cat were studied during a 2—2 5 h hemorrhagic hypotension using a plethysmographic technique The constrictor fibres were intermittently stimulated at 4 Hz before during and after a hypotensive period at approximately 60 or 40 mm Hg The nervous responsiveness of the resistance vessels and of the capacitance vessels declined continuously throughout the hypotensive period the rate of decline being higher the lower the perfusion pressure and the lower the arterial blood pH The capillary filtration coefficient (CFC) reflecting the tonus of the precapillary sphincters was increased during the hypotensive period Nervous activation reduced CFC throughout the experiments Tissue volume increased continuously during the latter half of most experiments at 40 mm Hg probably reflecting a deranged Starling equilibrium across the capillary wall During the post hypotensive control period after a 40 mm Hg hypotension arterial blood pressure fell rapidly Mucosal ulcerations were regularly found in these animals and the pathogenesis of these lesions is tentatively discussed

Many techniques have been used in the study of experimental shock (see *e g* Wiggers 1950 p 134 Swan and Nelson 1971) One of the most common models originally described by Werle Cosby and Wiggers (1942) is usually referred to as The Wiggers model Here hemorrhagic shock is produced by keeping the animal at a constant low blood pressure (30—50 mm Hg) by bleeding into a pressure bottle Utilizing such techniques intestinal hemodynamics have been investigated during hemorrhagic hypotension For example resistance vascular responses have been studied by Reynell *et al* (1955) Cull *et al* (1956) Selkurt and Brecher (1956) Friedman (1961) Longerbeam *et al* (1962) Lillihei *et al* (1964) and those of the capacitance vessels by *e g* Reynell *et al* (1955) and Johnson and

Selkurt (1958) using indicator dilution techniques or continuous weight measurements Further Wiggers *et al* (1946) Cull *et al* (1956) and Cock *et al* (1971) tried by measuring changes in portal vein pressure to follow reactions within the veins

However in no study have all the intestinal consecutive vascular sections been concomitantly and continuously followed during hemorrhagic hypotension particularly not as regards the impact of vasoconstrictor fibres on these during the course of prolonged hypotension The present study was performed to elucidate how such a competition between nervous and local chemical vascular control affects intestinal hemodynamics For this purpose blood pressure was kept at 60 or 40 mm Hg for 2—2 5 h by means of a modified Wiggers technique and the intestinal vascular bed was investigated by a previously described combination of a direct recording of flow and tissue volume (Folkow *et al* 1963) This allowed a comparison of the present results with those of previous studies where the small intestine was only regionally *exposed to prolonged hypotension (Haglund and Lundgren 1972 a b) corresponding* data are also available for cat skeletal muscle (Mellander and Lewis 1963)

Furthermore the effect of pH on intestinal vascular reactivity was also explored by artificially keeping arterial pH normal in one group of animals otherwise treated equally as the other animals

Part of this study was presented in a preliminary form (Haglund and Lundgren 1972 c)

Methods

A Operative procedures and determination of blood flow Experiments were performed on 29 cats weighing 2—5 kg and anesthetized i v with chloralose (50 mg/kg b w) after ether induction The details of the operative technique used were presented elsewhere (Folkow *et al* 1963 Haglund and Lundgren 1972a) and is only summarized here A jejunal segment of 30—40 g was chosen and the remainder of the intestinal tract extirpated The draining mesenteric vein was cannulated and connected to a drop recorder operating an ordinate writer venous outflow pressure being kept at 10—11 mm Hg to minimize passive venous collaps (Öberg 1967) Arterial pressure was recorded from the left femoral artery and arterial samples for pH P_{CO} and P_{O} measurements were taken from a cannula in the right femoral artery All splanchnic nerves were cut and their distal ends placed on ring electrodes for stimulation (4 Hz 6 ms 6 V) with a Grass stimulator (model SE) The adrenals were tied off and 10 mg cortisone acetate (Upjohn) was given i m The vagal fibres to the small intestine were left intact but in some experiments atropine (1 mg/kg b w) was given i v

B Plethysmographic technique The jejunal segment and its lymph nodes were enclosed in a perspex plethysmograph filled with Tyrode s solution (see Haglund and Lundgren 1972 a also concerning CFC determinations) Since venous outflow pressure was kept around 10 mm Hg mean capillary pressure was assumed to be 20 mm Hg for CFC calculations (Haglund and Lundgren 1972 b)

C The acid base balance of the experimental animal In most cats an i v infusion of 0 1—0 15 ml/min of a 10 per cent glucose solution containing 20 meqv $NaHCO_3$ per 100 ml was given to minimize the effect of the surgical trauma on acid base balance arterial and intestinal venous blood pH being repeatedly determined (Astrup pH meter 27 with a microelectrode unit) Arterial and intestinal venous blood P_{CO} and P_{O} were also followed in some experiments

D Bleeding technique The cats were bled into a specially designed plastic bag (Pedatrol Travenol lab Inc Morton Grove Illinois USA) preventing contacts between shed blood and glass By adjusting the bag position above the cat arterial pressure could be set at

TABLE I Arterial and intestinal venous blood pH before during and after a hemorrhagic hypotension at 40 mm Hg lasting on an average 126 min Mean value±S E n = 8

	Control		Hypotensive period				Control	
Time min			5	45	80	115	3	45
Arterial blood pH	7 34 ± 0 03	7 35 ± 0 02	7 51 ± 0 02	7 28 ± 0 04	7 18 ± 0 03	7 16 ± 0 04	7 05 ± 0 03	7 21 ± 0 03
Intestinal venous blood pH	7 25 ± 0 02	7 27 ± 0 01	7 25 ± 0 03	7 04 ± 0 04	7 01 ± 0 03	7 03 ± 0 03	7 00 ± 0 03	7 15 ± 0 03

desired levels upon bleeding Maximal bleeding volume was reached the first 30—60 min being about 25 per cent in the 60 mm Hg series and about 35 per cent in the 40 mm Hg series of the estimated blood volume (5 5 per cent of b w Farnsworth *et al* 1960 Aarseth and Bø 1972) During the last hour of hypotension a slow retransfusion to the cat was necessary to keep blood pressure at the desired level amounting to about 30 per cent of the shed volume at both pressure levels After about 2 h hypotension the remaining blood was returned i v after heating to body temperature

E Radioactive isotope technique In three experiments cat red cells were labelled with $Na^{51}CrO_4$ (AB Atomenergi Studsvik Sweden) according to Owen (1959) The ^{51}Cr emission was recorded by a detector so collimated as to register activity mainly from the tissues in the plethysmograph Thus changes in regional blood volume and tissue volume could be concomitantly and independently recorded

F Experimental procedures After enclosing the intestine in the plethysmograph the cat was allowed to rest for 30 min to reach a steady state as to arterial pressure intestinal blood flow and volume The sympathetic vasoconstrictor fibres were then stimulated for 20 min After a new poststimulatory control period blood pressure was reduced to 60 or to 40 mm Hg by bleeding By means of the bleeding device pressure was then kept at the desired level for 120—14? min During this hypotension the vasoconstrictor fibres were again stimulated at ??lar intervals for three 20 min periods During the subsequent post hypotensive control phase of 60 min another 20 min period of stimulation was performed with addition of a subsequent stimulation lasting only 3—4 min at the end of the experiment

Results

Experiments were performed at two arterial pressure levels 40 and 60 mm Hg The results obtained at 40 mm Hg will be described in detail while the 60 mm Hg experiments will only be commented upon as far as they differ from the results of the lower perfusion pressure

A Prolonged hemorrhagic hypotension at 40 mm Hg In this series of experiments 16 animals were used but five died during the severe hypotension In three experiments intestinal blood volume and tissue volume were simultaneously recorded (Methods section E) and these experiments are not included in Fig 1 and 2 and in Table I which hence are based on 8 expts

All of these animals received a slow bicarbonate infusion (Method section C) Arterial and intestinal venous blood pH were determined intermittently and the results are summarized in Table I Arterial blood pH increased during the early

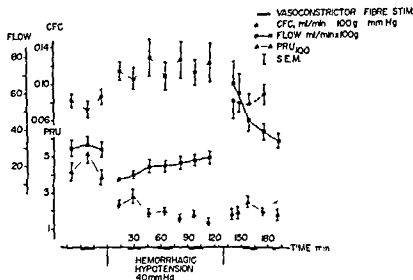


Fig 1 The effects of a prolonged hemorrhagic hypotension (arterial inflow pressure approximately 40 mm Hg) on blood flow regional flow resistance and capillary filtration coefficient (CFC) of the small intestine The regional sympathetic vasoconstrictor fibres were intermittently stimulated (6 V 6 ms 4 Hz) as indicated by bars on the abscissa Note that the flow and resistance values during nervous vasoconstriction depicted in the figure were registered during the steady state phase of sympathetic vasoconstriction (n = 8)

hypotensive phase in both series due to reflex hyperventilation reflected in arterial blood as a reduced P_{CO_2} and an increased P_{O_2} Despite the bicarbonate infusion arterial pH decreased continuously during the hypotension After retransfusion arterial and intestinal venous pH decreased markedly suggesting a hidden tissue acidosis that was unmasked by the improved perfusion

1 Resistance vessels Fig 1 shows the effects of hemorrhagic hypotension at 40 mm Hg on intestinal blood flow resistance and capillary filtration coefficient (CFC) The vasoconstrictor fibres were intermittently stimulated as indicated by black bars on the abscissa and the values given represent the steady state phase of neurogenic constriction (Folkow *et al* 1964 a b) Constrictor fibre stimulation produced no change in the steady state blood flow during control but flow resistance increased reflected as a pressure rise Upon bleeding the cats to the 40 mm Hg level resistance was markedly decreased In the early phase of hypotension constrictor fibre stimulation increased steady state resistance while in late phases only a small change was recorded Blood flow increased and flow resistance fell gradually throughout the hypotensive period In fact in some experiments blood flow even exceeded control late during the hypotension Retransfusion led to a longstanding marked hyperemia and flow resistance remained low during the posthypotensive observation period

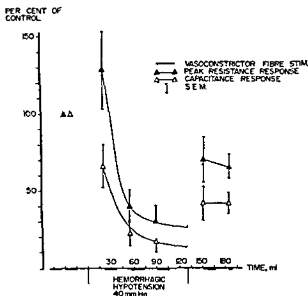


Fig 2 The effects of regional vasoconstrictor fibre activation on the intestinal resistance (peak resistance response) and capacitance vessels during and after a prolonged reduction of arterial inflow pressure to about 40 mm Hg Hypotension was induced by bleeding the animals The vascular responses are expressed in per cent of control value (n = 8)

Fig 2 illustrates the effect of 40 mm Hg hypotension on the peak resistance response to vasoconstrictor fibre stimulation (Folkow *et al* 1964 a Haglund and Lundgren 1972 b) expressed in per cent of control In the early hypotensive phase it exceeded control but subsequently it rapidly declined Vascular reactivity never recovered upon retransfusion

2 *Exchange vessels* CFC increased about 25 per cent in the early phase of the hypotension and then remained fairly constant (Fig 1) During the hypotension it was still possible to reduce CFC by constrictor fibre stimulation but it usually stayed above prehypotensive control level even during vasoconstriction Upon transfusion CFC returned to control

3 *Mean capillary hydrostatic pressure* Vasoconstrictor fibre activation caused in no instance any significant change of mean capillary hydrostatic pressure as compared to prestimulatory control Upon bleeding the animals to an arterial pressure of 40 mm Hg intestinal tissue volume decreased during the first 5—10 min Since this volume reduction parallelled the recorded reduction of regional blood volume no transcapillary net fluid exchange appeared to occur Later a gradual tissue volume augmentation was sometimes observed in the face of an unaltered regional blood content This volume increase was further accelerated during the period after retransfusion

Assuming that the continuous tissue volume increase during late hypotension and in the posthypotensive period was solely caused by transcapillary filtration these volume augmentations would correspond to capillary pressure increases of 1—3 and

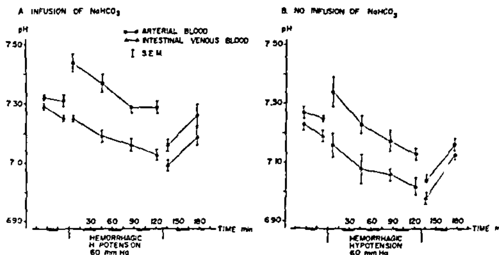


Fig 3 The alterations in pH of arterial blood and of intestinal venous blood before during and after a prolonged hemorrhagic hypotension at around 60 mm Hg 2 groups of animals were studied 5 cats were given an i v bicarbonate infusion (panel A) while another group of 5 animals served as controls (panel B)

4—9 mm Hg respectively 40—100 per cent of this fluid loss from the intestinal vascular bed was recovered in the intestinal lumen no luminal fluid being present in those animals where no regional fluid accumulation had occurred

4 Capacitance vessels During control the neurogenic capacitance response amounted to 2.8 ± 0.3 ml/100 g (mean $\pm$ S E n = 8) corresponding to some 40 per cent reduction of regional blood content Upon bleeding to 40 mm H tissue volume decreased 2.3 ± 0.3 ml/100 g during the first few minutes essentially due to a reduction in regional blood volume to judge from the experiments using radioactively labelled red cells The neurogenic capacitance response was markedly reduced during hypotension ultimately down to 15—20 per cent of control (Fig 2)

5 General posthypotensive condition of the animals In the posthypotensive period the cardiovascular system deranged progressively, as indicated by a 50 mm Hg fall in arterial pressure in 60 min during which one animal died Macroscopic postmortem examinations showed hemorrhagic lesions of the intestinal mucosa varying from disseminated petechial bleedings to large hemorrhagic areas

B Prolonged hemorrhagic hypotension at 60 mm Hg These experiments were divided into two groups Five animals were given in i v infusion of bicarbonate (Methods, section C) which kept arterial blood pH largely normal throughout To five other animals no such infusion was given thus allowing a gradual reduction of arterial blood pH The right panel of Fig 3 shows pH changes in arterial and intestinal venous blood before during and after 2 h of hemorrhagic hypotension at

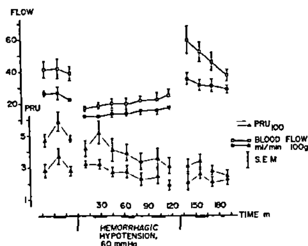
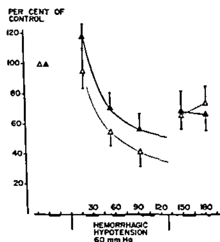


Fig 4 The effects of a prolonged reduction of arterial inflow pressure to approximately 60 mm Hg on blood flow and flow resistance in the small intestine To one group of animals (n = 5) bicarbonate was given i v (filled symbols) and another group (n = 5) served as control (open symbols) Hypotension was induced by hemorrhage Note that blood flow and flow resistance values during nervous vasoconstriction (black bar on abscissa) depicted in the figure were registered during the steady state phase of sympathetic vasoconstriction



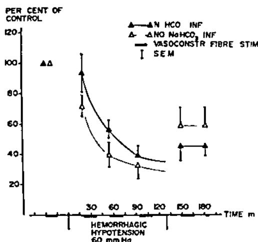


Fig 5 The effects of regional vasoconstrictor fibre activation on the intestinal resistance (peak resistance response) and capacitance vessels during and after a prolonged reduction of arterial inflow pressure to about 60 mm Hg The figure is based on the same experiments as in Fig 4 The vascular responses are expressed in per cent of control values

60 mm Hg as compared with the effects when bicarbonate was infused throughout (left panel) Comparing Fig 3 and Table I it is apparent that the pH changes were generally similar at the two pressure levels

The results obtained during a 60 mm Hg hemorrhagic hypotension are summarized in Fig 4 (blood flow and flow resistance) and 5 (nervous responsiveness

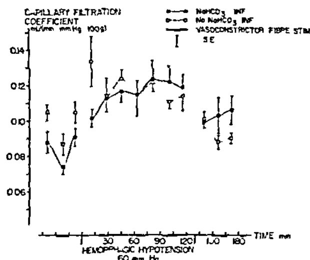


Fig 6 The intestinal capillary filtration coefficient (CFC) before during and after hemorrhagic hypotension at 60 mm Hg To one group of animals (n = 5) bicarbonate was given iv (filled symbols) and another group (n = 5) served as control (open symbols)

of resistance and capitance vessels) and in Fig 6 (CFC) Generally speaking the results obtained at the 2 pressure levels were qualitatively similar but quantitative differences were recorded The induced changes were often more pronounced during and after the lower perfusion pressure as *e g* noted for the decrease of flow resistance (Fig 1 and 4) and the decline of vascular reactivity to neurogenic vasoconstriction (Fig 2 and 5) Only transient tissue volume increases were observed in the post hypotensive periods of the 60 mm Hg expts

The animals receiving a bicarbonate infusion exhibited a lower blood flow and CFC as well as a higher peripheral resistance as compared to the acidotic cats Furthermore the vascular nervous responsiveness seemed to decrease faster during acidosis The general pattern was otherwise the same in the 2 groups of animals

The animals appeared to tolerate a 2 h period of hemorrhagic 60 mm Hg hypotension rather well Arterial blood pressure fell about 25 mm Hg during the 1 hour posthypotensive observation period. No animal died during or after the hypotensive period and mucosal bleedings were not observed at macroscopic postmortem examinations

Discussion

This study of the cat intestinal vascular bed during hemorrhagic hypotension was aimed at analyzing in detail the competition between neurogenic vasoconstrictor effects and vasodilating metabolites since the gastrointestinal circuit often appears to be particularly much compromised during shock. The results concerning each consecutive vascular section will be discussed separately below

The resistance vessels dilated rapidly upon severe hemorrhagic hypotension

probably mainly an expression of myogenic autoregulation (*cf* Haglund and Lundgren 1972 a) During prolonged hypotension the resistance vessels dilated further and blood flow reached within 2 h of 40 mm Hg hypotension almost control level Concomittantly with this the vascular responsiveness to constrictor fibres influences declined only to be partly restored by retransfusion These characteristics were more accentuated the more profound the hemorrhagic hypotension and it is probably a result of the progressive metabolite accumulation (see also Haglund and Lundgren 1972 b) The failure of the resistance vessels to return to control upon retransfusion furthermore suggests that the smooth vasculature was irreversibly damaged

In the literature intestinal blood flow has by some authors been reported to increase progressively during hemorrhagic hypotension (Reynell *et al* 1955 Cull *et al* 1956 Selkurt and Brecher 1956) in agreement with the present observations while others (Frideman 1961 Longerbeam *et al* 1962 Lillihei *et al* 1964 1967) reported slight reductions Upon retransfusion some authors (Cull *et al* 1956 Selkurt and Brecher 1956) report flow augmentations above control (*cf* Fig 1) while Frideman (1961) did not observe such a hyperemia During the subsequent posthypotensive hour all investigators report a declining flow as also recorded in this study It is then appearent that the intestinal vascular response pattern during hemorrhagic hypotension is largely similar in innervated and denervated preparations which is not surprising since vascular responsiveness to neurogenic influences declines fast during hypotension (Fig 2 and 5)

The precapillary sphincter activity is reflected by CFC measurements which evaluate in a semiquantitative way the surface area available for transcapillary exchange CFC increases during the first 0 5—1 h of hypotension probably mainly due to the accumulating metabolites (*cf* Haglund and Lundgren 1972 a) and constrictor fibre is then only seldom able to decrease CFC below control This observation argues against the suggestion by Lillihei *et al* (1964 1967) that intestinal ischemia *per se* might explain the mucosal ulcerations Average flow is fairly well maintained and the exchange surface increased according to the present results This does however not rule out that there might be an uneven flow distribution and that this is accentuated by the constrictor fibre influence making some areas relatively ischemic others overperfused

The neurogenic capaitance responses of the intestine declined during hemorrhagic hypotension even at a faster rate than was the case with the corresponding responses of the resistance vessels The intestinal capacitance vessels thus respond differently from those of the skeletal muscle (Mellander and Lewis 1963) This decline is probably explained by the accumulation of tissue metabolites (*cf* Haglund and Lundgren 1972 b) It may in the intact organism lead to considerable pooling of blood in the intestinal veins during hemorrhage If it is assumed that approximately 400 ml of blood can be expelled from the splanchnic area of man during intense neurogenic constriction (*cf* Folkow and Neil 1971) the results presented in Fig 2 would suggest that some 350 ml blood may again be pooled during

prolonged hypotension as a result of declining neurogenic responses

Splanchnic pooling of blood has been discussed extensively and is by some authors considered to be of great importance for the "irreversibility of shock Such a pooling has usually been inferred from increases of portal venous pressure during hypotension (Wiggers *et al* 1946 Cull *et al* 1956, Selkurt and Brecher 1956 Johnson and Selkurt 1958) reflecting an increased intrahepatic resistance and/or a failing heart Such a factor was ruled out in the present experiments since venous outflow pressure was held constant (*cf* Haglund and Lundgren 1972 a) Furthermore it was directly shown with the aid of radioactively labelled red cells that intestinal blood volume remained unaltered between stimulation periods during the hypotension Thus while the regional blood content did not change intestinal tissue volume increased gradually during severe hypotension particularly in the post hypotensive period By exclusion this must reflect an extravascular fluid accumulation which was mainly found in the intestinal lumen

The mechanisms causing this tissue volume augmentation also observed by Johnson and Selkurt (1958) have been earlier discussed (Haglund and Lundgren 1972 a b) Here edema and/or secretion could be involved It seems however less plausible that secretion an energy consuming process should be increased when blood flow was most markedly reduced *i e* at an arterial inflow pressure of 40 mm Hg Furthermore it was not possible to abolish the tissue volume increases by the administration of atropine Edema could be caused by a deranged Starling equilibrium across the capillary wall In earlier publications (Haglund and Lundgren 1972 a b) the edema was solely discussed in terms of a raised capillary hydrostatic pressure but in view of the epithelial sloughing from the villous tips observed in the mucosal ulcerations (Ahren and Haglund to be published) a locally disturbed colloid osmotic pressure equilibrium may well contribute The increased CFC may also reflect an altered capillary permeability but so far no clearcut evidence exists suggesting that low blood flow and the resulting hypoxia will affect capillary permeability (Korner 1959 Scott *et al* 1967)

Cook *et al* (1971) proposed that hypotension changed the intestines from an absorptive organ to a secretory one However these authors suggest that secretion should be a result of an increased capillary pressure and hydraulic conductivity of the intestinal epithelium together with a loss of intestinal ability to effectively pump sodium Thus the mechanism proposed by Cook *et al* is in reality a matter of a disturbed Starling equilibrium

In the present study the effects of blood pH on the response of the intestinal circulation to a 60 mm Hg hemorrhagic hypotension was investigated showing that almost throughout intestinal blood flow and CFC were higher and vascular responsiveness to neurogenic influences lower at a lower pH probably due to the well known vasodilator effects of the hydrogen ion (*cf* Bygdeman 1963) However upon retransfusing the animals no difference was noted between the two groups of animals as regards vascular tone or vascular reactivity to nervous stimulation

The effects of a strictly regional intestinal hypotension recently studied by Hag

lund and Lundgren (1972 b) were qualitatively similar to those reported here for hemorrhagic hypotension though some quantitative differences were found General ly speaking the regional hemodynamic changes in the intestine were as expected more pronounced when hypotension was caused by hemorrhage than when caused by local arterial obstruction

Mellander and Lewis (1963) investigated the effects of hemorrhagic hypotension on the consecutive vascular sections of cat skeletal muscle under experimental con ditions closely similar to the present ones As arterial pressure was lowered to 40—50 mm Hg skeletal muscle flow resistance was decreased 10—20 per cent of control and then remained largely unchanged during 2 5 h of hypotension Upon retransfusion resistance vascular tone was rapidly restored to normal CFC increased within minutes about 100 per cent and was then unaltered throughout the hypotension period only to be rapidly restored to control upon retransfusion The present results (*e g* Fig 1) illustrates the strikingly different response pattern in the intestinal vascular bed A comparison between these 2 major circuits also suggests that in con trast to the skeletal muscle the intestinal vasculature is severely damaged by pro- longed hypotension since its tone or reactivity were never reestablished upon re transfusion (see above)

Mellander and Lewis (1963) furthermore demonstrated that in skeletal muscle the neurogenic influence was better maintained in the postcapillary capacitance sec tion than in the precapillary resistance one eventually leading to an increase of mean capillary hydrostatic pressure upon nervous stimulation and to a capillary fluid filtration A corresponding type of mean capillary pressure increase was never ob served in the intestine as a result of a lowered perfusion pressure (Haglund and Lundgren 1972 b) This is probably explained by the equally rapid decline of nervous responsiveness of the resistance and the capacitance vessels in this tissue (Fig 2 and 5) It follows that in the small intestine mean hydrostatic capillary pres sure tends to be kept constant at the expen e of blood capacity blood pooling is here preferred to outward filtration This seems to be of advantage because with a capillary surface area at least 10 times bigger than in the skeletal muscle (Folkow *et al* 1963) a fluid loss caused by filtration could easily exceed that caused by blood pooling within the intestine Nevertheless at severe hypotension there often occurs independent of neurogenic influences a gradual and rather substantial fluid loss across the intestinal capillary walls

The hemorrhagic mucosal lesions reported in this and in a preceeding paper (Hag lund and Lundgren 1972 b) have earlier been described after prolonged hypotension in dogs by *e g* Wiggers (1950) Lillihei (1957) and Chiu *et al* (1970) and also in man (Penner and Bernheim 1939 Ming 1965 Sorensen and Vetner 1969) and in rats (Bacalzo *et al* 1971) Lillihei *et al* (1964 1967) discuss at length the pathogenesis of these mucosal ulcerations and according to him two mechanisms are of prime importance First he believes that the pre to postcapillary resistance ratio is decreased as described above for skeletal muscle (Mellander and Lewis 19 leading to intestinal edema that impairs blood tissue exchange However

chain of events could not be demonstrated either in this or an earlier study (Haglund and Lundgren 1972 b) Second Lillihei *et al* propose that a reduced mucosal blood flow, *as a result of sympathetic vasoconstrictor activity combined with a low* perfusion pressure, should cause hypoxia and eventually mucosal lesions However neither neurogenic vasoconstriction nor arterial hypotension at around 40 mm Hg will significantly reduce villous blood flow in the cat (Lundgren and Svanvik to be published) calling for other types of explanations

A countercurrent exchange mechanism was described by Lundgren (1967) in the hairpin vascular loops of the intestinal villi Such a mechanism implies that *e g* oxygen tension is low in the villous tips due to the extravascular short circuiting of the gas at the bases of the villi During hypotension the intestinal countercurrent exchanger probably becomes even more efficient, since mean transit time increases in the hairpin loops from 5 to 10—15 s as arterial blood pressure is lowered to 40 mm Hg (Lundgren and Svanvik to be published) Since this greatly increases the chances of extravascular oxygen shortcircuiting it might result in a degree of villous hypoxia severe enough to cause the cell death in their tips, as actually observed during low pressure perfusion of the gut (Chiu *et al* 1970 Ahren and Haglund to be published) Such a mechanism was proposed by Lundgren (1967) and discussed at some length by Chiu *et al* (1970) in connection with experiments performed at low intestinal blood flow

After severe hemorrhagic hypotension a general derangement of the cardiovascular system was observed in the present study after transfusion as also was the case during *regional* intestinal hypotension at 30 mm Hg (Haglund and Lundgren 1972 b) The role of the splanchnic area for the development of this deranged state has been intensely discussed after that Lillihei (1957) proposed the existence of an intestinal factor in irreversible shock (see *e g* Selkurt 1970) The intestinal fluid loss occurring in the present experiments could by no means alone by responsible for the cardiovascular derangement since it never exceeded some 5 per cent of the cat's blood volume It is therefore likely that a release of vasoactive substances occurs from the splanchnic area as discussed by *e g* Lillihei (1957) and Lefer (1970) It is however, in the present experiments very unlikely that the pancreas could be a major source for such substances as proposed by Lefer (1970) since here 3/4 of the pancreas was extirpated and was moreover not exposed to reduced flow when the effects of local intestinal hypotension was studied (Haglund and Lundgren 1972 a b) Nevertheless also these latter experiments a rapid cardiovascular deterioration occurred after prolonged but strictly regional intestinal hypotension This draws attention to the possibility that substances with toxic effecs are released from the intestine itself perhaps from the damaged mucosal epithelium

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Acta physiol scand 1973 88 109—122

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Influence of Fixation on Physiological Properties of Frog Gastric Mucosa

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Abstract

HELANDER H F W S REHM and S S SANDERS *Influence of fixation on physiological properties of frog gastric mucosa* Acta physiol scand 1973 *88* 109—122

Functional properties of the *in vitro* frog gastric mucosa were studied during fixation with glutaraldehyde (GA) or OsO_4 The acid secretory rate dropped quickly and was completely abolished within 5—15 min When fixative was added to either the serosal or mucosal surface of the mucosa the potential difference decreased rapidly within the first minute and frequently became temporarily inverted The electrical resistance changes almost always included an initial drop With GA added to either side and with OsO_4 added to the serosal side the resistance then rose to values above the controls again returning to very low values after intervals usually between 15 min and 2 h With OsO_4 added to the mucosal side the resistance decreased rapidly to low levels and did not recover The selective permeability of the nutrient membrane of K over Na was decreased within 5 to 10 min but was not abolished until about 35 min after the addition of the fixatives at which time the mucosal resistance was still high It is concluded that the ion-conducting pathways in the plasma membranes are greatly changed by the fixatives which should be taken into consideration when interpreting electron micrographs

Extensive studies have been performed on the physiological and biophysical properties of the *in vitro* amphibian gastric mucosa (*e g* Hogben 1955 Rehm 1966 Shoemaker Hirschowitz and Sachs 1967 Forte 1971) The morphology of this organ has also been studied in considerable detail both by light and electron microscopy (Sedar 1965 Forte Limlomwongse and Forte 1969 Geuze 1971 Helander *et al* 1972) In attempting to correlate morphological and functional findings problems arise concerning the changes produced in the tissue by the process of fixation In the present study the effects of fixation on the functional characteristics of the frog s gastric mucosa are reported Preliminary results have been published elsewhere (Helander Sanders and Rehm 1972)

Materials and Methods

Frog (*Rana pipiens*) gastric mucosa stripped of its external muscle layers was mounted between two lucite chambers in a manner previously described (Rehm 1962) The standard solution bathing the tela submucosa (nutrient or serosal solution) contained (in mM) Na 102 K 4 Ca 1 0 Mg^{++} 0 8 Cl 81 0 HCO_3 25 phosphate 1, SO_4 0 8 glucose 10 and histamine 0 1 In some experiments a high K serosal solution was used with the same composition except that K was 79 mM and Na 27 mM The solution bathing the surface of the mucosa (secretory solution) contained (in mM) Na 100 K 4 and Cl 104 Both sides were gassed with 95 % O_2—5 % CO_2 Two pairs of electrodes were used one pair for sending current across the mucosa and the other for measuring the transmucosal potential difference (PD) The PD was measured by means of a recording potentiometer and the resistance was determined by sending pulses of direct current of about 0 5 s duration across the mucosa (resistance = $\Delta PD/I$ Noyes and Rehm 1970) The resistances reported in this paper are ohms for 1 3 cm^2 (the area of mucosa in the chamber) The secretory rate was measured with a recording pH stat technique

After an initial control period OsO_4 or glutaraldehyde (GA) was added to either the secretory or nutrient solution or both As a rule 1 ml of fixative was added to about 9 ml of bathing solution Fixatives used were either 5 % OsO_4 in distilled water or 10 % vacuum distilled (Fahimi and Drochmans 1965) glutaraldehyde mixed with 1 % H_2O_2 (Peracchia Mittler and Frenk 1970) in distilled water The final concentration of OsO_4 was thus 0 5 % and of glutaraldehyde 1 % Liquid junction potentials were determined between the standard solutions and the same solutions after the addition of fixative by a previously described method (Hokin and Rehm 1947) They were found to be less than 2 mV

Some of the mucosae were processed for electron microscopy The tissue fixed at room temperature in the chamber for 3 h in glutaraldehyde was rinsed in frog Ringer's solution postfixed in ice cold 1 % OsO_4 (dissolved in the same medium) rinsed again contrasted in uranyl acetate dehydrated and embedded in Araldite or Epon The tissue fixed in OsO_4 for 1 h in the chamber was rinsed contrasted dehydrated and embedded in the same way

Ultramicrotome sections were stained with uranyl acetate and lead hydroxide and examined in a Philips EM 200 or EM 300 electron microscope One micron thick sections from the same blocks were examined by light microscopy after staining with toluidine blue pyronine or with the PAS method

Results

ctional Observations

Fig 1—3 show experiments on 6 different mucosae where H rate potential difference (mv) and resistance (ohms) are plotted against time At the time shown the fixative as specified was added to either the secretory or nutrient solution or to both solutions simultaneously

Secretory Rate

During the control periods the rates of H ion secretion were about 4 $\mu Eq\ h^{-1}\ cm$ When either OsO_4 or glutaraldehyde were added to either the secretory or nutrient solutions or to both sides simultaneously the H secretory rate as determined by our method (pH stat) dropped quickly and was completely abolished within 5—15 min When a substance that rapidly inhibits the H rate is added to the secretory side there is some uncertainty concerning the precise time course of the inhibition However in these experiments the H rate following addition of the fixative to the secretory side appeared to drop at least as rapidly as in the experiments in which the fixative was added only to the nutrient side

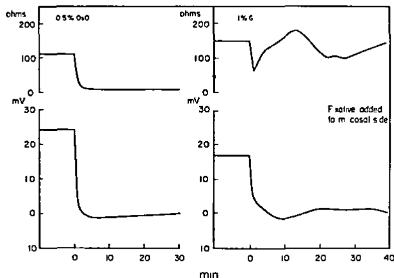


Fig 1

Potential Difference

The potential difference (PD) during the control period was usually between 15 and 25 mV with nutrient side positive When either O_sO_4 or GA were added to either side or simultaneously to both sides the PD dropped rapidly during the first minute (Fig 1—3) In some experiments with GA added to the secretory solution the PD following a rapid initial decrease leveled off for a few minutes at a value definitely above zero and then decreased to about zero In some cases the PD in

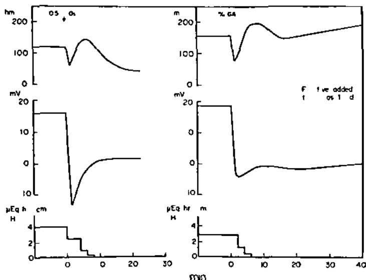


Fig 2

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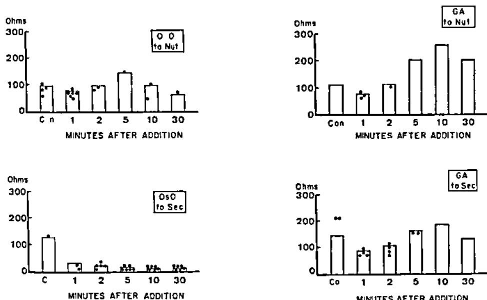


Fig 5—6 Tissue resistances (ohms for 1 3 cm²) at intervals after adding the fixatives GA to either side or OsO_4 added to the nutrient (serosal) side caused an increased or unchanged resistance whereas OsO_4 added to the secretory (mucosal) side resulted in decreased resistance

Resistance

The effects of the fixatives on the resistance of the tissue are seen in individual experiments (Fig 1—3) and the results are summarized in Fig 5 and 6 The addition of OsO_4 to the secretory side resulted in a rapid drop of the tissue resistance to very low values without much subsequent change The addition of OsO_4 to both sides simultaneously gave results essentially the same as OsO_4 to the secretory side alone OsO_4 to the nutrient or GA to either side resulted in an initial transient drop in resistance followed by a rise to above control levels which in some experiments was sustained for long periods In those experiments where the parameters were followed throughout the entire fixation the resistance was still above control levels after 2 h in glutaraldehyde At the end of the third hour in GA however the resistance in some cases had fallen to below control and subsequently after one hour in Ringers the resistance was at very low levels and then the addition of OsO_4 had no effect

Effects of OsO_4 on K Selectivity of Nutrient Membrane

Normally the nutrient membrane (*i e* the plasma membrane facing the nutrient solution) has a high selectivity for K over Na via conductance channels as determined by the response of the PD to changes in the K concentration of the nutrient fluid (K replacing the Na) (Harris and Edelman 1964 Spangler and Rehm

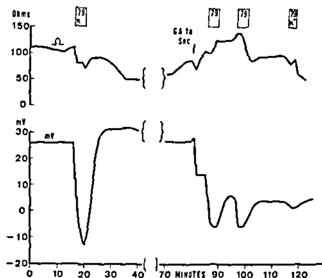


Fig 7 Effects of GA added to secretory (mucosal) side on selective K^+ permeability of the nutrient membrane K^+ concentration of nutrient solution elevated from 4 to 79 mM for time indicated by width of the blocks Increased K^+ concentration caused a ΔPD of about 40 mV Return to normal 4 mM K^+ brought PD back to near control levels After addition of GA to secretory (mucosal) solution (arrow) subsequent exposures of nutrient membrane to 79 mM K^+ caused ΔPDs of about 21 mV 11 mV and 2 5 mV

1968) An increase of nutrient K^+ from 4 mM to 79 mM results in a change in PD and the magnitude of this change is a measure of the relative conductance of the nutrient membrane to K^+ (Spangler and Rehm 1968) Fig 7 shows the effect of GA added to the secretory solution on the magnitude of this change in PD In this experiment the K^+ concentration in the nutrient solution was changed from 4 to 79 mM for the time indicated by the width of the boxes Before the fixative was added the ΔPD in 4 min was about 40 mV The nutrient solutions was then changed back to the regular 4 mK K^+ concentration and the PD rapidly returned to above the control level and then gradually declined to about the original level GA added to the secretory solution at the time shown by the arrow produced a fall in PD and a small temporary fall in the resistance followed by an increase Five minutes after the *addition of GA the nutrient K^+ is again elevated from 4 to 79 mM for 4 min* and the change in PD is about 21 mV On returning to control solutions the PD increased by about 11 mV 8 min later another pulse of high K^+ gave a ΔPD of 11 mV Finally 35 min from the time of the addition of the fixative to the secretory side the elevation of K^+ in the nutrient fluid gave a ΔPD of only 2 5 mV which is not significantly different in orientation and/or magnitude from the value for the liquid junction potential between the 4 K^+ and 79 solutions (Spangler and Rehm 1968) During the period of determining the effect of elevation of the K^+ on the nutrient membrane the resistance of the tissue was not less than that of the control level Similar experiments with 79 mM K^+ pulses were performed with OsO_4 added



Fig 8 Light micrograph of frog gastric mucosa The total thickness of the mucosa is about 0 3 mm Surface epithelial cells characterized by their dense secretory granules cover the surface of the mucosa and the gastric pits (P) Below the pits the epithelium consists mainly of oxyntic cells C gastric cavity L lumen of gastric gland MM muscularis mucosae OC oxyntic cell Epon Toluidine blue ×250

to the secretory solution alone to the nutrient solution alone and GA added to the nutrient solution alone In all of the experiments (a total of 8) the controls showed the typical response of the PD to elevation of the K in the nutrient solution and there was a marked decline in the response following the addition of the fixative with a reduction of the PD response to that of the liquid junction potential (about +2 5 *mV) within 30 min*

Morphological Observations

Light microscopy revealed gastric mucosae of normal appearance (Fig 8) Studies by electron microscopy showed oxyntic cells (Fig 9 and 10) surface epithelial cells (Fig 11) and mucous neck cells of essentially the same morphology as described in earlier publications (Sedar 1965 Forte *et al* 1969 Geuze 1971 Helander *et al* 1972) In addition a few epithelial cells were observed resembling the argyrophil cells in mammalian gastric mucosa

No systematic investigation was carried out to compare the quality of fixation between glutaraldehyde and OsO_4 fixed tissues nor between tissues fixed from the



Fig 9 Electron micrograph of oxyntic cell fixed in chamber by adding GA to both sides simultaneously Except for the vacuolization of occasional mitochondria the preservation is of adequate quality Numerous microvilli (V) project into the lumen (L) of the gland Extensive interdigitations are observed along the lateral cell borders (ID) A large number of tubules and vacuoles (T) are seen in the cytoplasm LY lysosome like body M mitochondrium N nucleus Araldite ×5800

mucosal side vs those fixed from the serosal side These methodological variations did not seem to cause any major difference in the ultra structure of the tissue However with both fixatives the apical cell membrane of the oxyntic cells was sometimes broken and some of the cytoplasmic ground substance then appeared to have floated into the gland lumen With OsO_4 these changes were generally more pronounced often resulting in a nearly total loss of the microvilli With glutaraldehyde on the other hand vacuolization of the mitochondria was a frequent finding

Discussion

One of the striking results of the present study is the speed with which the two fixatives act on this tissue The PD and resistance both undergo marked changes within the first minute and the H rate decreases within a few minutes There are diffusion barriers between the mucosal cells and the external bathing media on the secretory side there is the mucous coat and on the nutrient side the barrier consists of the lamina propria muscularis mucosa and part of the tela submucosa (Helander



Fig 10 Oxyntic cell fixed in chamber by adding OsO_4 to both sides The apical portion of the cytoplasm appears swollen bulging into the gland lumen (L) No microvilli are seen A large number of mitochondria (M) and vacuoles (V) dominate the cytoplasm N cell nucleus Epon ×7000

et al 1972 Spangler and Rehm 1968) The thickness of the nutrient barrier is about 0 25 mm and the time constant for the diffusion of small molecules through this barrier is about 1 min (Spangler and Rehm 1968) On the basis of the assumption that the rate of diffusion through the barrier is much greater than the rate through plasma membranes then the concentration of fixative at the nutrient membrane of the mucosal cell layer would be about 63 % of its concentration in the bathing solution in about 1 min (Spangler and Rehm 1968) This latter calculation assumes no appreciable sinks for the fixative in the barrier However the presence of sinks (regions that adsorb and/or absorb the fixatives) in the barrier should not greatly modify the rate at which the fixative accumulates at the mucosal cell border

It has been previously shown that any emfs in the cells in the nutrient diffusion barrier cannot contribute significantly to the overall PD (Spangler and Rehm 1968) they would be short circuited via the surrounding interstitial fluid Therefore it is apparent that the fixatives (molecular weight for OsO_4 is 254 and for glutar



Fig 11 Surface epithelial cells from crest of mucosal fold fixed in chamber by adding GA to both sides A few microvilli project from the apical cell surface into the gastric cavity (C) Below this surface there is an accumulation of densely packed secretory granules (G) The nucleus (N) is found in the basal portion of the cells Along the lateral cell borders there are marked interdigitations (ID) between the neighboring cells D desmosome ER rough endoplasmic reticulum M mitochondrium. Araldite ×3500

aldehyde 100) can diffuse relatively rapidly through both the secretory and nutrient barrier since the initial decrease in PD must be due to a change in the membranes of the mucosal cell layer

This rapid rate of diffusion of fixatives seems to be contrary to the results of the studies on other tissue For example immersion of liver blocks for 1 h in 1 % OsO_4 in distilled water produced a black zone of fixed tissue about 0 7 mm thick (Hagstrom and Bahr 1960) Treatment of slices of this tissue with thiourea revealed no unreduced OsO_4 deeper in the tissue block Similarly when liver blocks were immersed in a buffered 6 25 % GA solution for 2 h subsequent staining of frozen tissue sections indicated a diffusion of the fixative to a depth of only 0 5 mm (Reale and Luciano 1970, see also Ericsson and Biberfeld 1967) The time course of the diffusion of fixatives seems to be much more rapid in the gastric mucosa than in the liver The most reasonable explanation for this difference is that the main diffusion pathway in the stomach is through the extracellular spaces while the diffusion pathway in the liver is mainly through cells

In the studies on liver the fixatives diffuse through roughly 1/2 mm in 2 h Assuming the diameters of the cells are about 25 μm then the fixative diffuses through approximately 20 cells or 40 plasma membranes in 2 h which would be about 6 min per cell or about 3 min per membrane Extrapolating these results to the gastric mucosa shows that it would require only a few minutes to penetrate the nutrient membrane and reach the secretory membrane (i e the plasma membrane facing the secretory solution) This is about the time taken for the H secretory rate to be reduced to zero when the fixatives are added to the nutrient side The almost immediate effect on the PD when the fixatives are added to either side indicates that the immediate change in the PD is undoubtedly due to the effect of the fixatives on the plasma membrane facing the side to which they were added

Resistance Changes

One of the questions of particular interest was whether these fixatives produce a very marked reduction in the resistance of the tissue i e whether they produced very leaky plasma membranes It will be recalled that OsO_4 added to the secretory side or to both sides simultaneously produced in a few minutes a decrease in the resistance to very low levels In contrast GA to either side or to both sides simultaneously produced an initial decrease in resistance which was followed by a sustained increase usually to above control levels One might anticipate that OsO_4 added to the nutrient side alone would result in a decrease of the resistance to a low level within a few minutes However this did not occur after a transient decrease the resistance increased usually to above control levels and remained there for some time before decreasing to low levels Although at present we have no adequate explanation for this finding it raises the possibility that the effect of a fixative on the characteristics of a tissue is a function of the side to which it is first applied

There are a number of aspects of the effect of the fixatives on resistance that are of interest For instance the decline in H rate to zero in most cases occurred when the resistance was substantially elevated above the initial level which argues against the possibility that the H rate continued but was masked by the diffusion of ions through leaky plasma membranes (*e g* HCO_3 from nutrient to secretory fluid and/or H in the reverse direction) The disruption of the H mechanism could be due to inactivation of essential enzymes and/or a disruption of the special arrangement of the enzyme systems in the membrane Previous work has shown that some enzyme systems are not inactivated completely by GA (Sabatini Bensch and Barrnett 1963) The finding that GA added to either side or to both sides or OsO_4 added to the nutrient side resulted in after an initial decrease in resistance an increase to a level usually above that of the controls indicates that the fixative did not result in disruption of a significant fraction of the plasma membranes Even if only a very small fraction of the area of a plasma membrane is disrupted (resulting in aqueous spaces extending through the membrane) the resistance across the membrane would drop to about zero because of its extreme thinness The resistance R (in ohms) is given by the well known equation

$$R = \varrho \frac{L}{A}$$

where ϱ is the specific resistance (ohm cm) L the thickness (cm) and A the area (cm^2) of the membrane The actual surface area of either the secretory or the nutrient plasma membranes of the frog gastric cells as determined by electron microscopy is about 200 times greater than the macroscopic flat area (Helander Sanders Rehm and Hirschowitz 1972) The magnitude of L is about 80×10^{8} cm For disrupted portions of the membrane ϱ would be about 100 ohm cm (*i e* the specific resistance of the aqueous fluids adjacent to the membranes) For a membrane resistance of one ohm the actual area disrupted would be about 8×10 cm or 4×10 % of the total secretory or nutrient membrane area During the periods of elevated resistance there is obviously essentially no disruption of the plasma membranes

The increase in resistance can be explained in part on the basis of our separate site theory of HCl secretion in which it is postulated that there are electrogenic Cl and H mechanisms (Rehm 1950 and 1972) in the secretory membrane and that the nutrient membrane has a high passive conductance for K and Cl Potent electrogenic pumps in the secretory membrane would result in a low resistance of this membrane during active secretion The potency of an electrogenic pump would be proportional to its resistance (Rehm 1972) Inhibition of these pumps would result in an increase in the resistance of the membrane provided the inhibition of the pumps did not result from disruption of the membrane which would then be occupied by aqueous solution

These results on the resistances are also of interest in light of the findings of Elbers (1966) on eggs of *Limnaea stagnalis L* When the eggs were exposed to OsO_4 the plasma membrane immediately became very leaky to ions while with GA the membrane remained tight An increase in membrane resistance has been calculated also in cardiac Purkinje fibers after exposure to aldehyde fixatives (Fozzard and Dominguez 1969) This was tentatively ascribed to a chemical effect on the membranes reducing their permeability to both Na and K

Changes in Potential Difference

In our experiments the fixatives produced a decreased of the potential difference eventually to zero This decrease in PD could be due to a change in the K Na selectivity of the nutrient membrane or to inactivation of the Cl and/or H mechanisms on the secretory membrane Inversion of the PD is difficult to explain as a change in the conductance properties of the membranes with the existing ion gradients then being responsible for the inverted PD However inversion could be caused by an inactivation of the Cl mechanism before inactivation of the H mechanism since the emf of the electrogenic Cl pump is oriented to make the nutrient side positive while the emf of the H pump is oriented to make the nutrient side negative

The Effect of the Fixatives on the K Na Selectivity

One of the interesting characteristics of many biological membranes is the marked selectivity via conductance pathways for K over other cations such as Na. Until recently it was not possible to duplicate the selectivity in non living membranes. However with the work of Eisenman and colleagues (1962) on ion selective glass membranes and then with the work on thin lipid membranes impregnated with certain antibiotics (Mueller *et al* 1963, Andreoli Tiefenberg and Tosteson 1967) ion selectivities were obtained which were comparable to those of biological membranes particularly the selectivity of K over Na. A model that we have been using for the K conductivity of the nutrient membrane of the gastric mucosa has been patterned after the valinomycin lipid membrane findings (Andreoli *et al* 1967) and this has enabled us to offer satisfactory tentative explanations for a variety of experimental findings (Spangler and Rehm 1968 Rehm Spangler and Sanders 1969 Pacific *et al* 1969)

It is possible that the nutrient membrane contains K selective molecules constrained to the membrane but free to move within the membrane which may be similar to ionophores like valinomycin (Pressman 1965) The cavities in these molecules would be only about 1 3 Å (Mayers and Urry 1972 personal communication from D W Urry 1972) in diameter and could not be resolved in the electron microscope On the other hand it is possible that the whole molecules might be observed if there were proper contrast since their diameter would be about 15 Å (Mayers and Urry 1972 personal communication from D W Urry 1972) However their number per unit area would be expected to be very small and so the chance of observing them would not be great Still if conditions were ideal we might be able to see them In pursuing this aspect of the problem one would prefer to be able to fix the tissue for electron microscopy in such a way as to preserve the K selectivity Changes occurring during the dehydration and embedding (*cf* Moretz Akers and Parson 1969) must obviously also be considered In attempting to visualize these structures in the electron microscope one should consider the use of other fixation and embedding procedures possibly including inert dehydration fixation cryo ultramicrotomy or negative staining procedures that might produce less changes in membrane characteristics

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From the Biophysics Institute Aarhus University Denmark

Mechanical Properties of Guinea Pig Taenia Coli Muscles

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Abstract

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Conditions have been established under which taenia coli muscles give consistent responses to electrical stimulation in experiments lasting up to 8 h Using a new definition of resting length different muscles show similar mechanical characteristics In agreement with previous investigations the mechanical behaviour of taenia coli is found to be qualitatively similar to vertebrate skeletal muscles Compared with frog sartorius (a) the active tension per unit area is similar (b) the maximum power output about 80 times less (c) 90 % of he maximum active tension is developed over a range of lengths about twice as great Experiments with iodoacetic acid indicate that a condition of rigor can exist in this muscle

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This paper describes the behaviour of *taenia coli* muscles under conditions where certain mechanical responses could be consistently reproduced The investigation was carried out in the course of work done in our laboratory to develop a method by which this muscle could be stimulated to produce consistent responses in X ray diffraction experiments lasting many hours We found that by using a c field stimulation (Csapo and Goodall 1954 Casteels 1964) at 1 min intervals the isometric response would diminish by less than 10 % in experiments lasting up to 8 h Having established this we decided to reinvestigate certain mechanical properties of the *taenia coli* because it seemed possible that under the new conditions the results would be more consistently repeatable than appeared to have been the case in previous experiments of which the most important ones are those reported by Aaberg and Axelsson (1965) and Mashima and Yoshida (1965) Our results did not differ substantially from their findings However we believe that the methods we have used for relaxing and stimulating the muscles represent an improvement This also applies to attempts to define the muscle s reference length We demonstrated that by referring to the length for which the resting tension in Ca free Krebs solution is 5 % of the maximum active tension the resting and active tension length curves obtained for different muscles are very similar

Our experiments were carried out at 23° C as we found it difficult to obtain consistent results at higher temperatures However from experiments which we have done at higher temperatures (and from similar experiments reported by the investigators referred to above) it appears that the muscle s mechanical properties at 23° C are similar to those at the physiological temperature of 37° C the main difference being that isotonic shortening is about twice as fast at the higher temperature

Methods

Preparation

Adult guinea pigs were used The animals were anesthetized with ether for about 15 min during which time they died The abdomen was opened and the caecum exposed A length of about 5 cm of the *taenia coli* muscle was then carefully dissected out the preparation being kept moist with Krebs solution at about 23 C

Cotton threads were tied to the muscle and it was loaded with a 0 5 g weight The muscle was then allowed to relax by being placed in Krebs solution at 4 C After about 30 min cotton threads were tied to the muscle at points about 4 cm apart

The muscle was now transferred to the recording cell and the cotton threads tied firmly to the supports Krebs solution at 23 C was circulated through the cell

Solutions

All solutions were adjusted to pH 7 3±0 1

(a) Krebs solution

We used a solution containing (in mM)

K 5 95 Na 137 47 Ca^{2} 2 49 Mg 1 19 Cl 134 11 HCO_3 15 48 H_2PO_4 1 19 glucose 11 5 To this was added 0 1 g per litre streptomycin The solution was aerated with a mixture of O_2 (97 %) and CO (3 %)

(b) Adrenaline solution

This was a Krebs solution containing 10^{-6} g ml^{-1} adrenaline

(c) Ca free solution

This was a Krebs solution in which the Ca^{2} was replaced by 2 mM EGTA (Ethyleneglycol bis β amino-ethylether N N tetracetate)

(d) Iodoacetic acid (IAA) solution

This was a Krebs solution containing 5 mM monoiodoacetic acid neutralised with NaOH It was aerated with N_2

Stimulation

a c field stimulation was applied through silver coil electrodes *The field strength was adjusted for each muscle at each particular muscle length to give a maximum repeatable response* This normally varied between 5 and 10 V cm^{-1} being greater when a muscle was stimulated at short lengths It was found that if supra maximal stimuli were given the initial response was slightly reduced and subsequent responses decreased further

Normally muscles were activated in Krebs solution by applying the a c stimulus for 15 s (at 23 C) or 4 s (at 37 C) every 60 s Two typical isometric responses are shown in Fig 1 *Under these conditions the maximum tension developed would normally fall by less than 10 % throughout experiments lasting about 8 h*

Recording cell

Muscles were suspended horizontally between the tension transducer arm and the servo arrangement support in the recording cell which had a capacity of 200 ml Using a thermostatically controlled heater pump solutions could be circulated through the cell from a 1 5 litre reservoir Both the cell and the reservoir could be aerated One of the stimulating electrodes was placed behind the tension transducer arm and the other on the servo arrangement support The cell was surrounded with a water jacket and had an angled floor to facilitate complete draining

Servo-arrangement

The servo arrangement enabled the recording of muscle activity to be either isometric or isotonic In the isometric case the reference voltage could be set either by a hand potentiometer or a low geared motor driven potentiometer In the isotonic case the reference voltage was provided by feedback from the tension recording equipment A servomotor (Fern teuergerate MGW 40) drove the muscle support through a rack and pinion arrangement The maximum support movement was 4 6 cm The servopotentiometer output and reference voltage

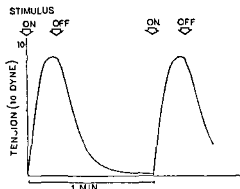


Fig 1

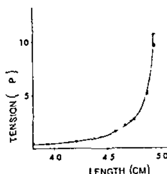


Fig 2

Fig 1 Isometric responses to 2 successive stimuli The muscle was stimulated for 15 s every 60 s using an a c stimulus of a field strength of 7 V cm $^{-1}$ Maximum tension developed 9 05 × 10 dyn corresponding to 1 72 × 10^{6} dyn cm Maximum rate of rise of tension 0 117 Po s $^{-1}$ Temperature 23 C Tracing of original record

Fig 2 Resting tension length curve Readings obtained in three consecutive runs indicated respectively ● ○ ■ □ ▼ Solid symbols indicate results while stretching and open symbols results while *releasing Mu cle in Ca free Krebs solution stretched between readings* at one muscle length per hour Readings taken about ten minutes after stretching/releasing was stopped when the tension reached a steady level l = 4 84 cm P1 = 8 4 × 10^{3} dyn Temperature 23 C

were connected to a difference amplifier (Fernsteurgerate Vtg 23) which fed the servomotor The compliance of the arrangement was small compared with the compliance of the tens on transducer

Record ng

(a) Tension

Semiconductor strain gauges (Kulite type D 350 O) were used for the construction of the tension transducer The output was linear over the work ng range The compliance with maximum tension development was about 1 of the muscle length This is substantially less than the series compliance of the muscle which has been estimated as about 13 (Aberg 1967) The output from the transducer was recorded on a potentiometer recorder (Goerz RE 511) having a maximum calibrated sensiti ity of 2 mV per 20 cm chart width The sensitivity of the arrang ment was about 800 dyn mV $^{-1}$ The zero dr ft was normally less than 0 2 mV in an 8 h experiment

(b) Length

Changes in *muscle length were measured using the output from the servo potentiometer* This was fed to another potentiometer reco der (Goerz RE 511) whose sensitivity as adjusted so that the whole movement of the muscle support corresponded to full recorder movement This gav a sens tivity of 4 35 cm recorder movement per cm support movement but this could be increased where necessary There was no detectable zero dr ft The muscle length was deter mined by d rect measurement in one position and thereafter from recorder readings

(c) Muscle mass

Aft r each experiment the muscle mass was determined by the follo ng p ocedure

The muscle was cut at the knots blotted firmly but without crushing it until no further moisture was seen to be e tracted and then immediately weighed

Dete mination of refer ce l ngth

The act e tension length and resting t ns on length experiments (see Re ults) have shown that the maximum acti e ten ion is obtained wh n the mu le i held nea a length equal to 85 of the length for which the resting t nsion is 5 % of the ma imum acti e t nsion We called the latter the *reference length* (l) and def ned the *wo k g length* (l_0) as 0 85 ls The act ve ten ion developed at l_0 has been denoted Po The reasons for using this m thod of def n ng

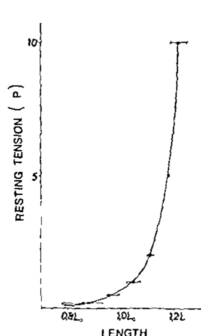


Fig 3

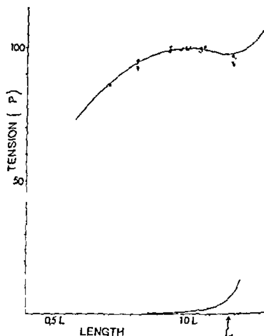


Fig 4

Fig 3 Resting tension length curve showing results from 8 expts Limits give standard deviation For definition of l_0 and l_0 see text Temperature 23 C

Fig 4 (a) Top total tension length curve showing results from 6 expts plotted ● ○ ■ □ ▼ ▽ (b) Bottom resting tension replotted from Fig 2 (c) Dotted active tensio nas the difference between (a) and (b) The points in (a) represent the average of the total tension developed in response to more than 20 stimuli at any particular muscle length Also shown ( ) are the results from three earlier experiments (unpublished) Temperature 23 C See text for explanation of l_0 and l

the resting length are given under Results At the start of all experiments (except those in which the resting tension length relationship was determined) this reference length was established by the following procedure

The mus le was stimulated at a length estimated to be its working length The response to sucessive stimuli usually first increased and then reached a steady value This value was taken to be an estimat of Po the tension developed at l_0

Adrenaline (10^{-6} g ml^{-1}) was then added and the muscle loaded isotonically with 0 05 times this estimate The muscle would then lengthen reaching a steady length after about 10 min This was taken as an estimate of l Next the adrenaline solution was replaced with Krebs solution and the length set to 0 85 of this estimate for l The muscle was then stimulated electrically If the final steady response differed from the initial response by more than 2 % the process was repeated The final estimate of l was taken as a measure of l_0 The shallow slope of the developed tension length curve near l_0 and the comparatively steep slope of the resting tension length curve at l_0 meant that the process did not usually have to be repeated

Criteria for the validity of an experiment

Results were only considered to be valid if the isometric response to electrical stimulation at length l_0 at the end of an experiment was about 90 % of the response at the beginning However in the experiments concerned with the resting tension length relation (where we were only interested in the non-contractile parallel elastic elements) we accepted experiments in which the final isometric response was 75 of the initial one

(A)

Results

Relaxing conditions

It is well known that the inherent spontaneous activity of the *taenia coli* presents an obstacle to a quantitative study of its mechanical properties In particular this activity makes it difficult to define the muscle s resting tension

Due to the presence of spontaneous activity we found it impossible to obtain consistent responses at temperatures around 37° C and we therefore worked at 23° C this being the highest temperature at which we could get repeatable results At this temperature spontaneous contractions occurred which could reach a peak tension level of 0 6 P_0

Means were investigated for suppressing such spontaneous activity at 23° C so that the *active tension* (the peak tension developed in Krebs solution in response to electrical stimulation) was subsequently unaffected

The following methods were tried

(a) Cooling

Below about 13° C spontaneous activity appeared almost completely inhibited (Bulbring and Kuriyama 1963 v Loh and Golenhofen 1970) However the muscle became much more viscous and this meant that it could take 1 h or more for the tension to become steady after an imposed length change

(b) Procaine

Mashima and Yoshida (1965) have reported that procaine causes *taenia coli* muscles to relax We found that by itself this drug had no effect unless the preservative chlorochresolum was present In the latter case however the muscle no longer responded to electrical stimulation on being replaced in a normal Krebs solution

(c) Adrenaline

Adrenaline in a concentration of $1 \; 10^{-6}$ g ml^{-1} is known to inhibit activity for periods up to 30 min at 23° C (Bulbring and Kuriyama 1963) The response to electrical stimulation in this solution was substantially unaffected as was the subsequent response in Krebs solution We found that further applications of adrenaline do not reduce spontaneous activity after it has restarted unless large doses ($1 \; 10^{-4}$ g ml^{-1}) are used in which case the subsequent response to electrical stimulation in Krebs solution is appreciably diminished

(d) Ca free solution

Spontaneous activity appeared to be completely and continuously inhibited in a Krebs solution where Ca had been replaced by EGTA (Bulbring and Tomita 1970) Furthermore there was no response to electrical stimulation Nevertheless even after immersion in this solution for many hours the subsequent active tension in Krebs solution was usually at least 75 % of the initial active tension and often nearly 100 %

The tension of a muscle in Ca free solution was therefore taken as the *resting tension* and this solution was used in determining the resting tension length relation However in other experiments where the reference length was routinely deter

mined adrenaline solution was used because this determination could be made within the time for which an adrenaline solution completely inhibits activity

Resting tension length relation

In determining the resting tension length relation the length changes between measurements were made very slowly at about one muscle length per hour, using the output from the low geared motor driven potentiometer as a reference voltage for the servo arrangement Measurements were made about 10 min after movement was stopped The results from each muscle were obtained from at least 2 stretch/release cycles At this rate there was no significant difference between measurements made whilst stretching or releasing (Fig 2) providing the muscle was not stretched beyond a length for which the resting tension exceeded about 0 1 P_0 at about 1 25 l_s For each muscle the active tension was also measured at a length for which the resting tension was 50 dyn This active tension (P_1) was measured at intervals throughout the experiment and if it fell below 75 % of the initial value the experiment was discontinued

The results from 8 muscles were examined to determine whether a way could be found to express the measurements for resting tension and length so that the resting tension length curves obtained for different muscles would be similar

The resting tension (P_R) was expressed as a proportion of P_1 or as a stress as given by the expression $\frac{P_R \rho l}{m}$ where ρ and m are the muscle density (taken as 1 g cm^{-3}) and mass The length (l) was expressed in turn as a proportion of the length for which (a) $P_R = 50$ dyn (b) $P_R = 500$ dyn (c) $P_R = 0\ 05\ P_1$ (d) $\frac{P_R \rho l}{m} =$ 10 dyn cm

It was found that expressing P_R as a proportion of P_1 and the length as a proportion of the length for which $P_R = 0\ 05\ P_1$ gave a correlation between muscles which was at least as good as any other combination, and we used it therefore as the basis for the procedure to determine the reference length as described above

In the active tension length experiments it was found that for any muscle P_1 is nearly equal to P_0 Hence the length for which $P_R = 0\ 05\ P_1$ is very close to l On this basis the results are shown in Fig 3 where the resting tension is expressed as a proportion of P_0 and the length as a proportion of l_0 (= 0 85 l)

From the slope of this curve the elastic modulus (as defined by Mashima and Yoshida (1965)) is 10 dyn cm at l_0 and about 2 10^6 dyn cm^{-2} at l_s

Tension developed at l_0

P_0 at 23° C was routinely determined For the 35 expts of our investigation it was found to be 9 5 10^3 dyn ± 22 % giving 2 0 10 dyn cm ± 19 %

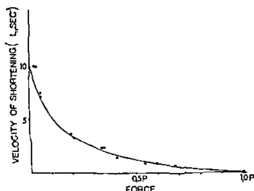


Fig 5

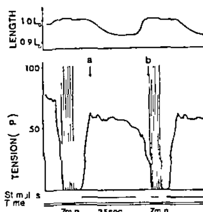


Fig 6

Fig 5 Force velocity curve showing results from 4 expts plotted ● ○ ■ □ Each point represents the average of two or three determinations at that load The curve is a hyperbola with asymptotes force = -0.17 Po and velocity of shortening = -0.017 $l_0 s^{-1}$ Temperature 23 C

Fig 6 Records of isotonic contraction starting at (a) Upper tracings show the change in muscle length For further details see text Same muscle as used in experiment described in Fig 1 Temperature 23 C Tracing of original record

The maximum rate of rise of tension was found to be 0 11 P $s^{-1} \pm 11$ % A tracing of 2 isometric contractions is shown in Fig 1 It should be noted that the stimulation is timed to stop just after the point where maximum tension is developed If stimulation is continued for longer than about 15 s the tension falls but does not drop below about 0 5 P_0 for several minutes

In 5 expts increasing the temperature from 23° C to 37° C caused the active tension to increase by (37 ± 20) % indicating a Q_{10} in this range of about 1 2

Below 23° C the tension developed and rate of rise of tension diminished with decreasing temperature At 4° C no response was detected

Active tension length relation

The active tension developed in response to electrical stimulation at 23° C was determined at muscle lengths ranging from 0 6 l_0 to 1 25 l_0 Six muscles were used in order to establish the width of the plateau over which the active tension remained fairly constant

We found that stimulating muscles outside the range of muscle lengths between 0 6 l_0 to 1 25 l_0 causes irreversible damage in that subsequent to such determinations it was no longer possible to obtain the same tension values at any of the points within the 0 6 l_0 to 1 25 l_0 range

The muscles were either stretched/released between readings at a rate of about one length per hour or stretched/released continuously at about one half length per hour In both cases the muscles were stimulated regularly throughout the experiment

for 15 s every 60 s The experiments lasted several hours during which time the muscles were stretched and released over the range 0 6 l_0 to 1 25 l_0 at least twice Each point shown in Fig 4 is the mean of between 20 to 50 responses, none of which differed by more than a few per cent Under these conditions there was no evidence for a shift in the curve along the horizontal axis in other words the phenomenon of plasticity was not observed

The resting tension curve shown in Fig 4 is redrawn from Fig 3 and the active tension at each muscle length is then taken as the total tension minus the resting tension at this point The active tension remained greater than 0 9 P_0 between 0 74 l_0 and 1 21 l_0 that is over a range of 0 47 l_0

Force Velocity relation

The force (tension) velocity relation was determined in 4 expts as follows The muscle was held at l_0 and regularly stimulated in the normal way (Fig 6) For each measurement the servo arrangement was switched to isotonic (at a in Fig 6) when the tension reached a certain preset level The comparatively slow response of the *taenia coli* enabled this to be done manually

During these contractions the stimulus duration was increased to 25 s after which further shortening ceased When the muscle had relaxed to length l_0 (at b in Fig 6) it was stimulated normally until the response to successive stimuli was constant over a period of at least 5 min If this response was less than 0 9 P_0 the experiment was discontinued

During each shortening phase the response time of the servo arrangement was sufficient to keep the tension within about 10 % of the preset level throughout the isotonic part of the contraction The relation between the maximum velocity of shortening which was maintained for about 10 s and the mean tension during this time was determined for a range of tensions between P_0 and 0 01 P_0 The results are shown in Fig 6 The points are found to lie close to hyperbolae for which Hill s (Hill 1938) constant a is 0 17 $P_0 \pm 30$ % and b is 0 017 l_0 $s^{-1} \pm 19$ %

In another series of experiments we determined the force velocity relation at both 23° C and 33° C using a different set up which included a mechanical release device At 23° C the constants a and b were then found to be 0 18 P_0 and 0 016 l_0 s^{-1} respectively while at 33° C a was 0 21 P_0 but b was 0 03 l_0 s^{-1} Thus the Q_{10} between 23° C and 33° C for a is 1 15 and for b 1 9

Effect of iodoacetic acid (IAA)

The average time course of the effect of IAA on four muscles is shown in Fig 7 The spontaneous activity was first inhibited and the tension then fell to the resting tension After that it increased reaching a maximum (0 12 $P_0 \pm 10$ %) about 1 1/4 h after the solution was changed from Krebs solution The tension then fell to the original resting tension within 2 h

In 1 expt we determined the elastic modulus at a point shortly after the tension reached its maximum and found it to be about 4 10^6 dyn cm This parameter is

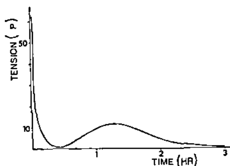


Fig 7

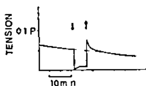


Fig 8

Fig 7 Time course of muscle tension after immersion in 5 mM iodoacetate solution Temperature 23 C Abscissa shows time after immersion in IAA solution

Fig 8 Determination of elastic modulus of an iodacetatic acid treated muscle At the arrow (↓) muscle was released 0 077 cm (0 019 l_0) while at the second arrow (↑) it was stretched to its original length Tension drop with reference to expected tension decay with no release (shown dotted) was 0 043 P_0 giving elastic modulus 4 10^6 dyn cm

however difficult to quantify because of the apparently viscous behaviour of the muscles in such a state A tracing of the tension record is shown in Fig 8

In another experiment a muscle was immersed in a solution containing only 1 mM IAA The time course was found to be similar to that observed with the 5 mM olution but the maximum tension developed was only 0 09 P_0

Discussion

Reference length

In order to compare the results obtained from experiments with different muscles a means of defining the length of each muscle has to be devised

As a criterion we decided that a definition for the reference length would be acceptable if by using it the resting tension length relation was found to be similar for different muscles This is met by our definition as described above and illustrated in Fig 3 Using our criterion we found the *in vivo* body length (Mashima and Yoshida 1965) not acceptable We also found that a reference length in terms of the relaxed length with a 50 dyn load (Aaberg and Axelsson 1965 Axelsson 1970) gives variable results possibly because the slope of the resting tension length curve at this point is such that small load changes cause large length changes Bulbring and Kuriyama (1963) have defined a reference length in terms of a particular weight to length ratio We found however that the variability in the size of the muscles we used make this also an unsuitable definition on our criterion

Resting tension length relation

The apparently complete absence of spontaneous activity in Ca free solution implies that in this condition little or no active tension is developed by the contractile

elements Thus the tension at each length may well be sustained entirely by the inert elastic elements in parallel with the contractile apparatus

A good deal of the confidence we have in our results is based on the marked lack of hysteresis in muscles which were stretched/released 2 or 3 times provided they were not stretched to a length for which the resting tension exceeded 0 1 P_0 and the rate of stretch did not exceed one muscle length per hour Fig 2 shows the results from one experiment and it will be seen that there is no difference between the 3 runs This behaviour is in contrast to that described as 'plastic by Axelsson (1970) who found a steady stress relaxation in stretched *taenia coli* muscles held in Ca free EGTA solution However in his experiments it appears that the muscles had been stretched to give a resting tension of about P_0 and this is well outside the range over which our observations were made

To check this point we have held muscles at lengths of 1 25 l_0 and 1 3 l_0 for several hours In the first 10 min after the muscles had been slowly stretched to these lengths there was a slight relaxation in the tension, but thereafter the tension remained steady at about 0 1 P_0 and 0 2 P_0 respectively On the other hand muscles stretched to give resting tensions of about 0 4 P_0 do show a stress relaxation phenomenon similar to that described by Axelsson

Our finding that adrenaline does not inhibit activity for more than about 30 min makes us wonder if the resting tension length curves obtained by other workers (Aaberg and Axelsson 1965 Mashima and Yoshida 1965) do in fact represent only the properties of the parallel elastic components In the light of our results it seems possible that their estimates of active tension may be too low particularly at lengths near l_0 In this case the maximum for their active tension length curves would be at too short a muscle length and their estimate of 1 would therefore also be too low This may explain why Aaberg and Axelsson found that a resting tension of 10^4 dyn is present at about 1 8 l_0 while under our conditions we found this value to occur at about 1 4 l_0

Muscles in which spontaneous activity has been suppressed by adrenaline or in a Ca free solution show distinct viscoelastic behaviour in that even with very slow tretching (one length per hour) some relaxation is seen when stretching stops On the available evidence it cannot be decided whether this viscosity is purely a property of the parallel elastic elements or due to some viscous or active interaction between the contractile elements

Active tension l_0

The present results suggest that our method of stimulation (even at 23° C) activa ed at least as many of the contractile elements as other methods which have been used We found the active tension at l_0 to be 9 6 10^3 dyn at 23° C and 14 4 10 dyn at 37° C, giving 2 0 10^6 dyn cm and 2 6 10^6 dyn cm respectively Using high K solution at 37° C Aaberg and Axelsson (1966) obtained 9 9 10^3 dyn active tension and 1 78 10^6 dyn cm Mashima and Yoshida (1966) obtained about 7 10^3 dyn active tension using a c field stimulation at 37° C In a recent paper Mashima and

Handa (1969) report an active tension of about $13 \cdot 10^3$ dyn for one muscle using a c field stimulation at 37° C We found that 10 % of our muscles stimulated at 23° C had an active tension at least as great as this

It may be noted that the active tension/unit muscle cross section we obtained is about the same as that found for frog sartorius striated muscle at 23° C

Active tension length relation

It is a striking fact that the *taenia coli* develops a very high level of tension over a considerable range of muscle lengths Although this tension was not found to remain constant over any part of the muscle length range it remained greater than 0 9 P_0 over a considerable wider range than the corresponding range for frog sartorius muscle We have called this the *90 % range* and as shown in Fig 4 found it is 0 47 l_0 for taenia coli We consider this value to be reliable because the active tension at each length did not change by more than a few per cent in any particular experiment each of which lasted several hours Our value for the 90 % range agrees substantially with the results of other investigators For example Aaberg and Axelsson (1965) (using high K solution) showed the 90 % range to be 0 55 l_0 although as already mentioned we think that their estimate of l_0 is too low In comparison the 90 % range for frog sartorius is about 0 22 l_0 (*e g* Deleze 1961) It is quite clear therefore that the *taenia coli* operates over an appreciably wider range of muscle lengths than the frog sartorius

Gordon and Siegman (1971) have recently published an active tension length curve for the rabbit *taenia coli* Using electrical stimulation they found that the 90 % range was only about 0 17 l_0 We have repeated these experiments and can confirm their results We have also obtained similar results using high K and acetylcholine stimulation The difference from guinea pig *taenia coli* muscles appears to arise because in the rabbit muscle the resting tension length curve is shifted to the left with reference to the total tension length curve The peak of the active tension length curves thus occurs at a point where there is already considerable resting tension When the muscle s contractile elements develop tension at this point it is probable that some of the parallel elastic components are relaxed slightly Thus because the resting tension is about 30 % of the total tension the difference between these two tensions may be significantly different from the true active tension This could account for the rabbit *taenia coli* having a shorter 90 % range even though the properties of its contractile apparatus may be similar to those of the guinea pig *taenia coli*

Force velocity relation

As for striated muscle the isotonic force and shortening velocity in *taenia coli* were found to be hyperbolically related Table I shows the results we and others have determined for a and b (Hill 1938) their Q_{10} values and the shortening velocity at zero load as given by the hyperbolic equation together with the corresponding values for frog sartorius It will be seen that as for striated mu cle a in *taenia coli*

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A good deal of the confidence we have in our results is based on the marked lack of hysteresis in muscles which were stretched/released 2 or 3 times, provided they were not stretched to a length for which the resting tension exceeded 0 1 P_0 and the rate of stretch did not exceed one muscle length per hour Fig 2 shows the results from one experiment and it will be seen that there is no difference between the 3 runs This behaviour is in contrast to that described as plastic by Axelsson (1970) who found a steady stress relaxation in stretched *taenia coli* muscles held in Ca free EGTA solution However in his experiments it appears that the muscles had been stretched to give a resting tension of about P_0 and this is well outside the range over which our observations were made

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TABLE I

Muscle	Temp	a	(Q_1)	b s^{-}	(Q)	$\frac{P b}{a^{-1}}$	Reference
Frog							
Sartorius	0 C	0 27 P		0 28 1		1 05 1	1
		0 25 P	(1)	0 33 1	([illegible] 05)	1 32 1	2
	23 C	0 25 P		1 41 1		5 64 1	3
Taenia							
coli	23 C	0 17 P		0 017 1		0 10 1	4
	23 C	0 18 P		0 016 1		0 09 1_0	5
	33 C	0 21 P	(1 15)	0 03 1	(1 9)	0 14 1	5
	36 C	0 17 P		0 05 1		0 30 1	6
	37 C	0 062 P		0 013 1_0		0 21 1	7

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1 Abott and Wilkie (1953)
2 Hill (1938)
3 By interpolation from Hill (1938)
4 This investigation
5 An earlier investigation of ours (unpublished)
6 Mashima and Handa (1969)
7 Aaberg and Axelsson (1965)

Comparison of dynamic constants (Hill 1938) for frog sartorius and taenia coli

It can be seen from Fig 9 that under conditions of zero load the *taenia coli* is capable of shortening to between 0 3 and 0 2 l_0 Whereas these isotonic responses were found to be reversible (in that the subsequently developed tension at length l_0 was unaffected) stimulation of a muscle held isometrically at such short lengths was found to reduce the subsequent active tension

Effect of IAA solution

The purpose of these experiments was to determine whether a condition of rigor could be produced in *taenia coli* muscles in view of previous findings that this could not be done in vascular smooth muscles (Lundholm and Mohme Lundholm 1966) Our results have shown that if the *taenia coli* muscles were held at l_0 in IAA solution the tension present (after first falling) increases to about 0 12 P_0 At this stage electrical stimulation of the IAA treated muscle produces no detectable response and its elastic modulus is about 40 times greater than that of a muscle held at the same length in Ca free solution The muscle s mechanical behaviour appears to resemble that seen in the rigor condition of striated muscle including the finding that the tension and elastic modulus revert to those of untreated muscles within a few hours (Sandow and Schneyer 1955) In striated muscle rigor is also accompanied by certain characteristic structural changes in the axial low angle X ray diffraction pattern (Huxley and Brown 1967) Such changes have yet to be demonstrated in *taenia coli* muscles

We wish to thank Mrs Karen Eskesen for technical assistance and Mr F Marquard for constructing the recording cell and servo arrangement
We are also grateful to Drs J C Haselgrove J V Small and P J Vibert for many useful discussions

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Acta physiol scand 1973 88 137—144

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Lack of Correlation between Profiles of Transmitter Efflux and of Muscular Contraction in Response to Nerve Stimulation in Isolated Guinea Pig Vas Deferens

By

LENNART STJARNE

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Abstract

STJARNE L *Lack of correlation between profiles of transmitter efflux and of muscular contraction in response to nerve stimulation in isolated guinea pig vas deferens* Acta physiol scand 1973 *88* 137—144

The profile of the nerve stimulation induced contractile response of isolated superfused guinea pig vas deferens was compared with that of transmitter efflux as monitored by determination of outflow of total tritium after preincubation with tritium labelled noradrenaline Transmitter efflux increased with time during stimulation up to 60 s and was monophasic although the contractile response could be made to shift from mono- to diphasic Desipramine and normetanephrine added to block local reuptake of noradrenaline altered the slope but not the monophasic character of the transmitter outflow curve Phenoxybenzamine abolished the delayed contractile response to nerve stimulation but potentiated and shortened the latency of the initial twitch In spite of the presence of drugs blocking reuptake of noradrenaline phenoxybenzamine further markedly enhanced nerve stimulation induced transmitter efflux indicating that blocking of specific α adrenoceptors leads to enhanced transmitter secretion Further addition of prostaglandin E_2 distinctly potentiated and prolonged the latency of the initial twitch but depressed the secretion of transmitter All these effects increased with the dose of prostaglandin and were reversible The results clearly show the sometimes striking lack of correlation between transmitter secretion and the resulting mechanical response actual determination of transmitter efflux improves on the accuracy of conclusions concerning mechanisms controlling secretory events at this level

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The isolated guinea pig vas deferens has been extensively used as a model system for the study of sympathetic neurotransmitter secretion As yet most conclusions concerning variations in transmitter secretion have not been based on direct determination of transmitter efflux but on indirect evidence the electrophysiological changes and the mechanical contraction induced by nerve stimulation

In the present paper it is shown that there is a marked lack of correlation between the profiles of transmitter efflux and of the resulting mechanical response induced by electrical nerve stimulation

Material and Methods

Vas deferens preparations from guinea pigs weighing 250—500 g were used for the experiments After decapitation of the animals the vasa deferentia were dissected out washed and incubated for 30 min at 30 C in one ml of Tyrode solution (NaCl 0 8 % KCl 0 02 % $CaCl_2$ 0 02 % MgCl 0 01 % $NaHCO_3$ 0 1 % NaH_2PO_4 0 005 % glucose 0 1 % sodium ascorbate 20 µg/ml and atropine sulphate 1µg/ml) aerated with 5 5 % CO_2 in O and containing *10 µCi of ^3H l noradrenaline (^3H l NA New England Nuclear Corp 6 5 Ci/mmole)* After repeated washing in isotope free Tyrode solution pieces of vas deferens about 10 mm in length were mounted in the superfusion bath described by Swedin (1971 a) Flow was maintained constant at 2 5 ml/min and the temperature was kept at 30 C The effluent was divided into fractions corresponding to 5—60 s periods and collected in an automatic fraction collector (Minirac LKB) Changes in the length of the preparation were recorded by means of an isotonic transducer (Harvard) loaded to balance tone recordings were made on a Grass Polygraph Nerve stimulation was performed by electrical field stimulation with *biphasic pulses of supramaximal voltage* and a duration of *1 5 ms* Trains of 300—600 stimuli were applied at 10 Hz with about 10 min intervals The stimulation parameters were checked on a Tektronix type OS 15A oscilloscope The field stimulation technique used has previously been shown to result in supramaximal nerve stimulation (Swedin 1971 a)

Aliquots of the effluent 0 15 ml were added to 10 ml of Instagel (Packard Instruments) and the radioactivity was determined in an ABAC SL 40 Intertechnique Liquid Scintillation Spectrometer by counting for 10 min

In this study the outflow of endogenous NA was monitored by determination of the *nerve stimulation induced rise in total radioactivity in the superfusate after previous labelling* of the neuronal NA stores by preincubation with a tracer dose of labelled NA Aluminium oxide and ion exchange chromatography indicated that more than 90 % of the radioactivity retained in the washed tissue was intact NA The rise in total radioactivity in the superfusate induced by nerve stimulation was assumed to represent material originally released from the nerves a intact NA For this reason no efforts were made to distinguish between intact NA and its metabolites in the superfusate

The following drugs were used Desipramine (DMI Pertofran® Geigy) normetanephrine (NMN Sigma) phenoxybenzamine (PBA Dibenzyline® Smith Kline & French) and prostaglandin E_2 (PGE_2)

Results

In the fresh preparation nerve stimulation at 10 Hz for 30 s resulted in a submaximal monophasic contraction On repeated stimulation at 1 min intervals the contraction changed and became increasingly diphasic with an initial rapid twitch followed after a variable latency by a delayed contraction However the nerve stimulation induced efflux of total ^3H assumed to reasonably well reflect total transmitter outflow was consistently monophasic at all times and progressively increased with time throughout the stimulation period of up to 60 s In all experiments the preparations gradually developed signs of mechanical fatigue towards the end of the stimulation period of 60 s in spite of continued rise in transmitter efflux (Fig 1)

Since the absence of an initial peak in transmitter efflux corresponding to the twitch might be due to rapid reuptake of transmitter the experiments were repeated after addition of drugs to block local reuptake of NA DMI was used to block Uptake 1 and NMN to block Uptake 2 (*cf* Iversen 1967) Maximal enhancement of nerve stimulation induced efflux of tritium was obtained at DMI 3×10^{-7} M further addition of NMN above 10^{-6} M slightly further enhanced ^3H efflux or nerve stimulation In order to ensure that reuptake of NA was safely blocked DMI 10^{-6} M and NMN 10^{-4} M were used in the experiments At these concentrations the drugs changed the diphasic contractile response into a mono-

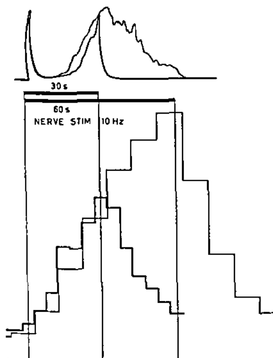


Fig 1

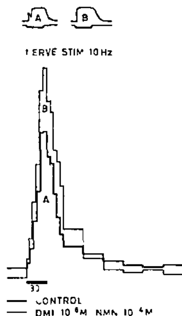


Fig 2

Fig 1 Nerve stimulation induced biphasic contractile response and essentially monophasic efflux of total ^3H in isolated superfused field stimulated guinea pig vas deferens

Fig 2 Nerve stimulation induced initially biphasic contractile response brought to re fuse by addition of DMI and NMN Monophasic efflux of total ^3H

phasic one and enhanced nerve stimulation induced tritium efflux However while the drugs made the slope of the efflux curve steeper the profile remained mono phasic no early peak corresponding to the twitch was seen (Fig 2)

On further repeated nerve stimulation the contractile response again changed into a diphasic one on prolonged stimulation up to 60 s there was often no sign of mechanical fading or fatigue in the delayed response in the presence of drugs blocking reuptake of NA (Fig 3) However on repeated stimulation signs of fatigue gradually appeared even in the presence of DMI and NMN

Addition of the α receptor blocking drug PBA at 7×10^{-7} M consistently blocked the delayed mechanical response to nerve stimulation but potentiated and shortened the latency of the initial twitch (Fig 4 B 2) Although the medium already contained supramaximal concentrations of drugs blocking reuptake of NA PBA always markedly enhanced nerve stimulation induced transmitter efflux (Fig 4 A) All these effects were more pronounced at PBA 7×10^{-6} M (Fig 4 A B 3)

Further addition of PGE at a progressively increasing concentration progressively enhanced the amplitude duration and latency of the twitch while on the other

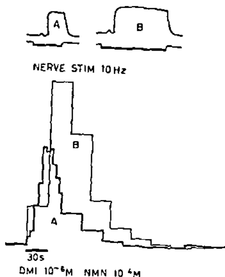


Fig 3 Nerve stimulation induced biphasic contractile response the delayed phase well maintained throughout the stimulation period of 60 s after addition of DMI and NMN (*cf* Fig 1 where these drugs were not present) monophasic efflux of total ^{3}H without clearcut delay corresponding to the delay of the small twitch Same preparation as in Fig 2 later during the experiment

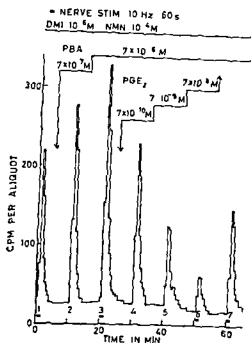


Fig 4 A

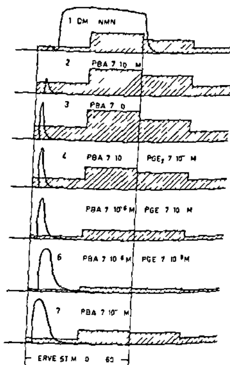


Fig 4 B

Fig 4 A Nerve stimulation induced efflux of total tritium DMI and NMN present throughout the experiment to block local rebinding of free NA Effect of PBA and PGE$_2$
Fig 4 B The mechanical recording and part of the ^{3}H efflux curve from the stimulations in Fig 4 A For further explanation see text

hand depressing the tritium efflux (Fig 4 A B 4—6) The effects were rapidly reversible within about 10—20 min (Fig 4 A B 7)

Discussion

The aim of the present study was to compare the time course and profile of nerve stimulation induced transmitter secretion with that of the resulting muscular contraction in the isolated guinea pig vas deferens

The method used for evaluation of transmitter secretion consisted in determination of the nerve stimulation induced rise in total ^{3}H in the superfusate after previous labelling of the neuronal NA stores by preincubation with tracer amounts of ^{3}H l NA This method is widely used as a means of monitoring secretion of endogenous transmitter in many tissues including the vas deferens (*e g* Langer 1970 Farnebo and Malmfors 1971 Hughes and Roth 1972) After blocking local rebinding of NA the nerve stimulation induced rise in total ^{3}H should give a reasonably good reflection of total secretion of endogenous NA This is probably true in spite of the observation that newly formed NA is preferentially secreted (Kopin *et al* 1968 Gewirtz and Kopin 1970 Stjarne and Wennmalm 1971) or that the time course of nerve stimulation induced efflux of newly formed NA differs from that of exogenous NA (Hughes and Roth 1972) suggesting that preformed endogenous NA may follow yet another time course The efflux rate of newly formed NA was found to reach a maximum after 40 s of stimulation while that of exogenous NA required 60—100 s (Hughes and Roth 1972) However during the initial 5—10 s of stimulation when the twitch response was observed it seems unlikely that preferential secretion of preformed endogenous transmitter could be so selective that it would show a rapid initial secretory burst while newly formed and exogenous NA slowly and progressively increase with time One rea on to doubt such a possibility is the electrophysiological evidence from guinea pig vas deferens which led Burnstock Holman and Kuriyama (1964) to conclude that transmitter secretion per nerve impulse probably increases with time during the course of a short stimulation period This is precisely the picture obtained in the present experiments using the efflux of exogenous NA as a monitor Interestingly the same time course has been observed for total nerve stimulation induced NA efflux as determined by bioassay in several isolated tissues (Hughes 1972)

The biphasic contractile response to nerve stimulation in vas deferens has recently been extensively discussed by Swedin (1971 b) and by Farnebo and Malm ors (1971) According to the present results the mechanical response whether monophasic or biphasic was at all times caused by an essentially monophasic secretion of NA starting from a low level and progressively and relatively linearly increasing with time up to 60 s The progressive rise with time in transmitter secretion per nerve impulse (*cf* Hughes 1972) and the resulting rise in NA concentration at the receptor level seem to be the cau e of the delayed contractile response The mechanical fading frequently seen during the later part of this phase was clearly not caused by

fading of transmitter secretion, but rather by failure in transmitter contraction coupling (cf Farnebo and Malmfors 1971)

As shown by Swedin (1971 b) the initial twitch is very probably adrenergically mediated in spite of the fact that it is not abolished, but rather potentiated by α adrenoceptor blocking agents (Bentley and Smith 1967) It seems likely that the twitch is initiated by a tightly coupled , highly efficient neuromuscular trans mission mechanism possibly involving junctional receptors (Hotta 1969) protected against e g drugs by their anatomical organization while the delayed response may be due to activation of less tightly coupled , less well protected extra junctional receptors as suggested by Swedin (1971 b)

The difficult part seems to be to explain the reversible splitting of the originally monophasic contractile response The observation that DMI and NMN tend to induce re fusion of the initial twitch to the delayed response suggests that the mechanical silence i e the decay of the twitch might be due to a local fall in the concentration of free NA caused by focal neuronal silence restricted to critical junctional areas while the average NA secretion throughout the preparation con tinues to rise with time Swedin (1971 c) suggested that the failure of the ' twitch to remain fused to the delayed response could be due to local accumulation of endogenous PGE triggered by the sudden initial contraction this would dramatical ly depress further secretion of NA as originally shown by Hedqvist (1969a) How ever although blocking of PGE formation with certain drugs counteracts the pro gressive fading of the contractile response to nerve stimulation in the vas deferens (Swedin 1971 c Hedqvist and Euler 1972 a) it does not prevent splitting of its originally monophasic profile

Focal neuronal silence in critical junctional areas could also be due to trigger ing of an α adrenoceptor mediated feed back control of NA secretion as originally proposed by Haggendal (1970) This hypothesis has received considerable experi mental support (Farnebo and Hamberger 1970 Langer 1970 Starke Montel and Schumann 1971 Wennmalm 1971 Enero et al 1972 Starke 1972) Recent observa tions made in experiments with guinea pig vas deferens indicate that this control mechanism is probably not dependent on local formation of PGE (Stjarne 1973 a b c)

DMI and NMN brought about fusion of the biphasic contractile response and also enhanced nerve stimulation induced efflux of ^3H indicating successful blocking of local reuptake of NA However further addition of PBA markedly further elevated transmitter efflux supporting the concept that an α receptor mediated mechanism normally restricts secretion of transmitter At the concentrations of PBA used the delayed mechanical response was abolished while there was a dose dependent potentation and a shortening of the latency of the initial twitch (cf Bentley and Smith 1967 Farnebo and Malmfors 1971 Swedin 1971 d)

In spite of the reasons to question the PGE dependence of the α receptor mediated mechanism apparently controlling transmitter secretion (Stjarne 1973 a b) exogenous PGE applied to the vas deferens was strikingly efficient in overcomin

the enhancing effect of PBA on transmitter secretion as first shown in the cat spleen by Hedqvist (1969 b) At the same time the amplitude and duration as well as the latency of the twitch were markedly increased (*cf* Hedqvist and Euler 1972 b) The results show that exogenous PGE depresses the slope of the nerve stimulation induced efflux of transmitter without altering its monophasic profile Similar depression of the local NA concentration is probably the reason why the closely related compound PGE_1 depressed excitatory junction potentials in isolated guinea pig vas deferens (Sjöstrand 1972) thus delays the summation leading to spike generation and subsequent contraction (Burnstock and Holman 1961) and thus explaines the observed increase in latency induced by PGE

The depression by exogenous PGE- of the PBA induced enhancement of transmitter secretion was manifest at PGE concentrations 1/10 000 of that of PBA Although the effect of PGE increased with the dose used the observation (Stjarne 1973 c) that it is independent of the concentration of α blocking drug indicates that the two agents do not have the same target in neuronal stimulation secretion coupling

The present experiments clearly show that the correlation between the profiles of nerve stimulation induced transmitter secretion and of the resulting muscle contraction is variable and may sometimes be very weak It appears safer to make inferences concerning transmitter secretion from electrophysiological studies of changes in the post junctional membrane However the present tracer method and preparation seem to offer a convenient means of supplementing the picture by actual determination of a parameter which provides a simple and efficient reflection of nerve stimulation induced secretion of endogenous sympathetic transmitter

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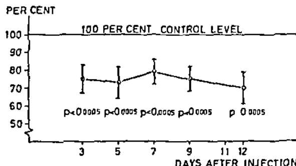


Fig 1 Concentration of noradrenaline per g wet weight of mouse kidney as per cent of control values at different times after intraperitoneal injection of Ehrlich ascites carcinoma Difference is highly significant ($p < 0\,0005$) at all times after injection

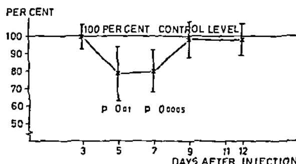


Fig 2 Concentration of noradrenaline per g wet weight of mouse kidney as per cent of control values at different times after subcutaneous injection of Ehrlich ascites carcinoma. Difference is significant ($p < 0\,01$) 5 days after injection and highly significant seven days after injection ($p < 0\,0005$)

Material and methods

33 female albino mice were injected i p with Ehrlich ascites carcinoma 36 were injected s c in the neck and 36 were used as intact controls Groups of animals were killed with chloroform at different time intervals 3 to 12 days after injection Part of the right kidney was used for histochemical analysis (Eranko 1967 Corrodi and Johnsson 1967) About 20 sections were prepared from each histochemical specimen and one of the authors subjectively graded the perivascular specific fluorescence of small kidney blood vessel (arterioles and intralobular arteries) on the following scale − = no fluorescence ± = fluorescence weak and seen in occasional blood vessels + = fluorescence weak but visible in most vessels + + = fluorescence strong and visible in all vessels In calculating the results each slide (animal) was considered as an independent observation The left kidney was used for biochemical tests as described earlier (Brownlee and Spriggs 1965 Laverty and Taylor 1968 Tallqvist *et al* 1970)

TABLE I Number of histochemical slides with different grades of subjectively estimated fluorescence of adrenergic perivascular nerves of small kidney blood vessel in mice with intraperitoneally and subcutaneously injected Ehrlich ascites carcinoma as compared with intact controls

Estimate of fluorescence	Number of slides/number of mice		
	Intraperitoneally injected mice	Subcutaneously injected mice	Controls
−OR±OR+	17/36	10/35	7/38
++	19/36	25/35	31/38
Difference compared with controls	$p < 0\,05$	Not significant	

Results

The histochemical results are shown in Table I There was no statistically significant difference between any two of the groups at any individual sampling time However if the observations from the whole experiment are pooled the i p injected mice had a greater number of slides with fluorescence estimated as negative or weak (— + or +) than the controls The difference is statistically significant $p < 0.05$ The s c injected animals did not differ significantly from the controls

The biochemical results are shown in Fig 1 and 2 The kidneys of the i p injected animals contained less noradrenaline than the controls The difference was highly significant at all time intervals after injection for both noradrenaline per kidney and noradrenaline per gram wet weight of kidney Differences between s c injected mice and controls were highly significant statistically ($p < 0.0005$) on the 7th day after injection and significant ($p < 0.01$) on the 5th day

Discussion

Since noradrenaline was depleted in the perivascular nerve fibres of the kidney when ascites was formed we thought that noradrenaline depletion might be related to the sodium and water retaining mechanisms (Tallqvist *et al* 1971) This hypothesis received further support from the fact that heart noradrenaline is reduced in heart failure with oedema and/or ascites in both experimental animals and humans (Vogel Jacobowitz and Chidsey 1969 Fizel and Fizelova 1969 Chidsey *et al* 1963 Chidsey Braunwald and Morrow 1965 Kramer Mason and Braunwald 1968 Mason *et al* 1970 Januszewicz and Wocial 1970) The reduction of heart noradrenaline in heart failure could be explained by defects in nordrenaline synthesis (Pool *et al* 1967) and in uptake and binding of catecholamines by cardiac sympathetic nerves (Spann *et al* 1969) Noradrenaline was also reduced in the blood vessels and left ventricle of dogs on sodium restricted diets (Hayduk *et al* 1970) In the edema ascites syndrome of cirrhosis the overall amount of potassium is decreased and so is the sodium concentration of plasma (Darnis 1971) In vitro potassium accelerates the synthesis of noradrenaline and lack of potassium or sodium blocks the transport of noradrenaline into isolated synaptosomes and further lack of potassium slightly increases the efflux of stored noradrenaline from isolated synaptosomes (Bogdanski Blaszkowski and Tissari 1970) It therefore seems to exist a theoretical basis for assuming that the noradrenaline depletion is correlated to mechanisms involved in the ascites formation

However we also found some noradrenaline depletion in mice with non ascite tumours but this was short lived and detectable only by biochemical means Reports reviewed earlier (Tallqvist *et al* 1971) described noradrenaline depletion in heart brain liver spleen and adrenal medulla in various states of stress thermal injury hypoxia or haemorrhagic hypotension Therefore since tumour growth is a non specific stress factor some noradrenaline depletion would be expected

We conclude that noradrenaline depletion in mice with ascites experimentally induced by Ehrlich ascites carcinoma is pricipally due to ascites formation Further experiments will elucidate the mechanisms of the phenomenon

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The Effect of Hypertonicity on the Time Course of the Active State in Single Skeletal Muscle Fibres of the Frog

By

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Abstract

Andersson K E *The effect of hypertonicity on the time course of the active state in single skeletal muscle fibres of the frog* Acta physiol scand 1973 *88* 149—159

The time course of the active state was investigated in single skeletal frog muscle fibres in normal Ringer's solution and in Ringer s solution made hypertonic (1 44—1 67 R) by addition of sucrose During a single isometric twitch the plateau phase of the active state was abbreviated in hypertonic solution During repetitive stimulation there was a progressive increase in the duration of the active state and a slowing of the rate of decay of the activity in the hypertonic medium. The resting membrane potential was insignificantly affected by the hypertonic solutions used There was a slight decrease in overshoot and also a diminution in the rate of rise and rate of decay of the action potential As a possible explanation of the results it is suggested that in a hypertonic medium there is a diminished release of calcium in response to a stimulus This leads to a shortening of the plateau phase of the active state during a single twitch Because of a reduced rate of re uptake of calcium by the sarcoplasmic reticulum the duration of the active state is prolonged and the decay of activity slowed during repetitive stimulation

As originally proposed by Hill (1938) muscle functions as an active contractile unit in series with a passive elastic element The response of the contractile unit to a stimulus the active state is characterized by an ability to produce tension or motion The intensity of the active state defined as the capacity of the contractile unit to produce tension at different times can be determined experimentally in single muscle fibres by using previously described approaches (Ritchie 1954 a b Edman 1970 Edman and Kiessling 1971)

During tetanic stimulation of skeletal muscle the maximum intensity of the active state is reached and maintained as long as the muscle is stimulated continuously This means that the plateau tension during an isometric tetanus is a convenient measure of the maximum intensity of the active state In a single twitch on the other hand tension development is time limited and peak tension is determined not only by the maximum intensity of the active state but also by the duration of the activity When the active state starts to decay the rate of isometric tension develop

ment is reduced At this point the isometric twitch myogram departs from that produced during a tetanus (Macpherson and Wilkie 1954) The maximum tension attained during a twitch will therefore be only a fraction of that reached during a tetanus

Previous studies on whole muscle and single fibres have shown that hypertonic solutions reduce both the isometric tetanic tension and the twitch response (Fenn 1936 Howarth 1958, Edman and Andersson 1968 Gordon and Godt 1970) This suggests that the maximum intensity of the active state is reduced The effect on the twitch is more pronounced than hat on the tetanus and at a tonicity where the twitch is abolished tension can still be produced in response to tetanic stimulation (Hodgkin and Horowicz 1957 Gordon and Godt 1970) The failure of a single stimulus to evoke a twitch under these conditions has been ascribed mainly to a lowering of the shortening velocity for this reason then the contractile unit is unable to stretch the series elastic element and to produce an appreciable amount of tension during the activity period (Howarth 1958) Another possible mechanism is that the duration of the active state is influenced by hypertonicity This possibility has so far not been thoroughly investigated The aim of the present investigation was therefore to study the effect of hypertonic solutions on the time course of the active state in single skeletal muscle fibres of the frog The effects on membrane potential and action potential were also studied

Methods

Preparation and mounting Single fibres were dissected from the ventral head of the semi tendinosus muscle of *Rana temporaria* A small portion (approx 0 5 mm wide and 2 mm long) of the tendons was left at each end In the tendons small rhomboid hooks of stainless steel (diameter 0 1 mm) were attached The fibres were mounted horizontally in a thermostated Perspex chamber between a glass tube extending from the anode peg of an RCA 5734 tension transducer and an isotonic lever Resting length and degree of shortening of the fibre could be controlled by means of two adjustable screws in front of and behind the lever A third stop connected to an electric relay was used for quick releases of the lever during active state recordings The relay was triggered by the stimulation signal and could be operated at preset times during contraction by means of an electronic delay unit This recording arrangement was similar to that described in detail by Edman and Kiessling (1971) Recording of fibre tension measurement of sarcomere length at rest and stimulation of the fibres were performed as previously described (Edman 1970 Andersson 1972) In some experiments the signal from the tension transducer was electrically differentiated This was done by feeding the transducer output into an RC circuit (time constant 1 ms)

Measurements of membrane potential and action potential Glass capillary electrode filled with 3 M KCl were used Their resistances were between 15 and 25 MΩ and their tip potentials were less than 5 mV The high input impedance amplifier (Holmer and Lindstrom 1972) used for the recordings had the following data input resistance $> 10^{12}$ Ω input current $< 10^{-12}$ A input capacitance < 0 2 pF (capacitance neutralization) and long term drift 0 2 mV/12 h The amplifier output was displayed on a Tektronix 502 A oscilloscope and photographed on 35 mm film (Scientia 135) by means of a Cossor oscilloscope camera The signal was also used to modulate an audio frequency generator A successful impalement was followed by a sharp change in frequency The rate of change of the action potential was recorded by electrical differentiation For this purpose the amplifier output was fed through an RC circuit (time constant 0 1 ms) and displayed on the oscilloscope screen

Solutions A Ringer's solution (normal Ringer's) of the following composition was used (mM) NaCl 115 5 KCl 2 0 $CaCl_2$ 1 8 NaH_2PO_4 Na_2HPO_4 2 0 pH 7 0 Hypertonic solutions were prepared by adding various amounts of solid sucrose to normal Ringer's solution (Table I) The tonicity of the solutions were routinely measured with a freezing point osmometer (Advanced Instruments Inc) In the following the hypertonic solutions used are denoted as 1 44 R 1 67 R etc

TABLE I Composition of the different hypertonic solutions

Solution	Approx osmolality mOsm/kg H_2O	Relative tonicity ×R
Normal Ringer's	225	1
Normal Ringer's +50 mM sucrose	275	1.22
Normal Ringer's +100 mM sucrose	325	1.44
Normal Ringer's +150 mM sucrose	375	1.67
Normal Ringer's +200 mM sucrose	425	1.89
Normal Ringer's +250 mM sucrose	475	2.11

All experiments were performed at 1.5—3.0 °C. The chemicals were of analytical grade.

Experimental procedure. The duration of the active state was determined by three different methods.

1. The decay phase of the active state was determined according to the modification of Ritchie's (1954 b) quick release method described by Edman (1970). The fibres were stimulated every 2 min at a frequency of 3—6 Hz to give 4 or 5 incompletely fused twitches. The peaks and troughs in the isometric myogram obtained in this way represent the tension output when the contractile unit and the series elastic element are in equilibrium and at a momentary standstill. By releasing the fibre at different times after the first stimulus, data points were obtained to define the decay phase of the active state in the 1st to 5th contraction cycles. In addition, points on the rising phase of the active state in the 2nd to 5th cycles were also obtained. A measure of the maximum intensity of the active state was obtained by the tetanic tension at the sarcomere length to which the fibre was allowed to shorten after release.

2. The duration of the plateau phase of the active state (= maximum intensity) was determined according to the method of Ritchie (1954 a). Fibres were stimulated every 3 min to produce a fused tetanus. The stimulation frequency was 25—40 Hz and the duration about 1 s. The sensitivity of the tension recording was increased to such a degree that changes of less than 1 mg could be detected. After a control period, the time from the last stimulus in a tetanus to the beginning of the decay of tension was measured. This time provides a measure of the duration of the plateau of the active state during the last cycle of activity.

3. The duration of the maximum intensity of the active state in a single twitch was determined according to the method described by Macpherson and Wilkie (1954). The fibres were stimulated every 2 min with a single shock to produce 8—10 twitches. When the twitches were exactly reproducible, two stimuli were given with an interval that was determined previously to give a fused ("tetanic") response. This series was repeated 2—4 times. The myogram from a single twitch was superimposed on the myogram obtained in response to 2 stimuli. The point where the two curves separate indicates the beginning of decay of the active state.

Before the beginning of the experiments and after every change of solution, the fibres were carefully paced, with the time intervals indicated above, for 15—30 min. Measurements were then made at a sarcomere length of 2.15 to 2.20 µm, first in normal Ringer's and then in hypertonic solution. At the end of the experiments, a series of control measurements was usually made in normal Ringer's solution.

The latency period was determined from the film records at 20× magnification in a Nikon comparator. The time was measured from the first stimulus to the point in the myogram where the mechanical activity could just be detected.

Results

Effects on twitch and tetanic tensions. Fig. 1 gives the mean value of twitch and tetanus tensions from 3 different single fibres in the hypertonic solutions used. As can be seen, *at a tonicity of 2.11 R there was no detectable twitch tension.* However, a tension of nearly 50 per cent of that obtained in normal Ringer's could be

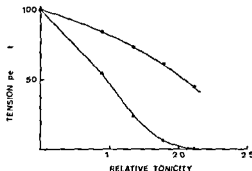


Fig 1 Effects of different hypertonic solutions on twitch tension (▲) and on tetanic tension (●) in 3 single fibres Abscissa relative tonicity Ordinate tension in per cent of the response in normal Ringers

produced in response to tetanic stimulation These findings agree with those obtained by Gordon and Godt (1970) in studies on bundles of muscle fibres

Most experiments were performed in 1 44 R In this solution the tetanic tension was reduced to 81 5 ± 1 1 per cent (mean and s e m) of the value obtained in normal Ringer s solution The amplitude of the twitch response was 45 9 ± 3 2 per cent of the control and the latency period was 21 5 ± 0 9 ms compared with 13 1 ± 0 6 ms in normal Ringer s It was found that both twitch and tetanus tensions were stable for at least 2 hours

The reversibility of the effects produced by 1 22—1 67 R was almost complete

Effects on the onset of decay of the active state In six single fibres the duration of the plateau phase of the active state during a twitch was determined according to Macpherson and Wilkie s (1954) method in both normal and hypertonic solutions (Table II) The latency period increased by about 8 ms in 1 44 R There was also a marked increase in the time to peak tension (mean 51 ms range 38—82 ms) No marked changes were found if the time from the first stimulus to the end of the plateau of the active state was measured but if a correction was made for the increased latency the active state was actually abbreviated (Table II) One of these

TABLE II Active state during a single twitch determined according to Macpherson and Wilkie (1954) in normal Ringer s solution and in 1 44 R (mean ± s e m n = 6)

1 Solution	2 Latency period ms	3 Time to peak tension ms	4 Time from first stimulus to the end of the active state plateau ms	5 Duration of the active state plateau (column 4 — column 2)	6 Difference in duration of active state plateau ms
Ringer s	13 0 ± 0 7	180 5 ± 18 7	49 3 ± 1 0	36 2 ± 1 4	7 5 ± 1 0* with n test error
1 44 R	21 5 ± 1 2	231 5 ± 19 1	50 2 ± 1 9	28 6 ± 2 1	

The difference in duration of the active state plateau between Ringer s and 1 44 R is highly significant (P < 0 001)

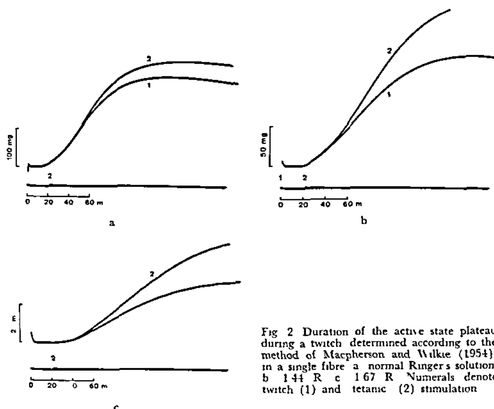


Fig 2 Duration of the active state plateau during a twitch determined according to the method of Macpherson and Wilkie (1954) in a single fibre a normal Ringer s solution b 1 44 R c 1 67 R Numerals denote twitch (1) and tetanic (2) stimulation

experiments where measurements were performed also in 1 67 R is illustrated in Fig 2 a b and c As the recordings show there was practically no difference in the time from the first stimulus to the separation of the twitch and tetanic curves in normal Ringer s and in 1 44 and 1 67 R However if a correction is made for the increased latency the duration is found to be shortened by approximately 9 and 17 ms in 1 44 and 1 67 R respectively

In some of these experiments the resolution of the mechanical recordings was increased by electrical differentiation of the tension record The same qualitative results were demonstrated but as the onset of activity could be resolved more precisely the times recorded with this technique were shorter

In 4 fibres the duration of the plateau phase of the active state after tetanic stimulation was determined by measuring the time from the last stimulus in a tetanus to the first sign of decay of tension In all fibres a prolongation of the maximum intensity was obtained in hypertonic solution (1 44 R) The increase varied from 48 to 98 per cent Fig 3 illustrates one of these experiments

Effects on the falling and rising phases of the active state In 12 fibres immersed in 1 44 R the falling phase of the active state was determined by quick release

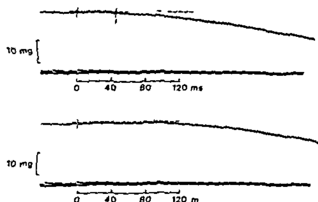


Fig 3 Duration of the plateau phase of the active state after tetanic stimulation determined according to the method of Ritchie (1954 a) Upper panel normal Ringer's solution Lower panel 1 44 R

technique The rate of decay of the active state was found to be decreased by repeated stimulation of the fibre This is illustrated in Fig 4 which shows the decay phases of the 2nd—5th contraction cycles in one of the experiments Before and after measurements in hypertonic solution, *determinations were also made in normal* Ringer's As can be seen the rate of decay of the active state was little influenced during the 2nd cycle However it became gradually slower through the 3rd to 5th *cycles As is also illustrated in Fig 4 the duration of the* active state *measured at* 50 per cent intensity level became progressively longer by repeated stimulation Three out of 6 fibres released in the 3rd contraction cycle showed a mean increase in duration of 19 per cent in the other 3 fibres no prolongation of the active state could be established With release in the 4th cycle a prolongation of 32 4 per cent was obtained (range 24 0—45 7 per cent n = 8) When the release was made in the 5th contraction cycle the duration of the active state increased by 48 2 per cent (mean of 3 fibres)

In the experiment illustrated in Fig 4 also the plateau phase of the active state after tetanic stimulation was determined It can be seen that the duration was almost doubled in 1 44 R hypertonic solution As indicated by extrapolation from the experimental active state curves *the results support the view that* there is a gradual increase in the active state plateau through the 3rd to 5th contraction cycles

TABLE III Resting membrane potential and action potential (AP) values in normal Ringer's solution and in 1 44 and 1 67 R (mean ± s e m)

Solution	Number of bundles	Number of fibres impaled	Resting membrane potential mV	Overshoot mV	Rate of rise of AP V/s	Rate of fall of AP V	Duration of AP at −50 mV, ms
Ringer's	6	21	90 8 ± 0 4	42 3 ± 0 4	97 0 ± 1 9	27 0 ± 0 5	6 9 ± 0 07
1 44 R	5	18	97 4 ± 0 8	34 1 ± 1	80 5 ± 2 8	22 7 ± 0 6	7 4 ± 0 11
1 67 R	5	18	92 8 ± 1 0	30 0 ± 0 6	74 9 ± 2 1	23 1 ± 0 5	7 4 ± 0 03

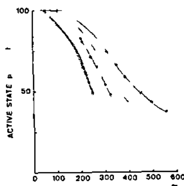


Fig 4

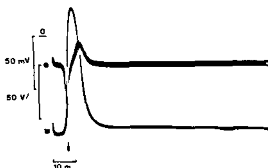


Fig 5

Fig 4 The decay phase of the active state determined according to Edman s (1970) quick release method in a single fibre Unfilled symbols normal Ringer s solution Releases were made in the 4th (O) and 3rd (△) contraction cycles respectively before and after the experiments in 1 44 R Filled symbols 1 44 R Releases were made in the 2nd (▲) 3rd (●) 4th (■) and 5th (★) contraction cycles The duration of the plateau phase of the active state after tetanic stimulation in normal Ringer s and in 1 44 R respectively is indicated by vertical bars at the 100 per cent level of active state intensity

Fig 5 Four superimposed action potentials in a fibre stimulated at intervals of 150 ms in 1 67 R Upper trace is dV/dt obtained by electrical differentiation

The effect of 1 44 R on the rising phase of the active state was studied in 6 fibres In both normal Ringer s and in 1 44 R the active state underwent a very rapid rise after the latency period as previously described (Edman 1970) No differences could be established between results obtained in normal Ringer s and in 1 44 R

Effects on membrane potential and action potential In small bundles of muscle fibres (3—5 fibres in each bundle) membrane potentials and action potentials were recorded in normal Ringer s and after immersion for 20—40 min in 1 44 and 1 67 R (Table III) The resting membrane potential was not markedly affected many fibres showed a hyperpolarization of 3—8 mV but this was not a constant finding and some fibres depolarized by a few mV The effects on the action potential by changing the tonicity varied quantitatively from fibre to fibre However immersion in hypertonic solution consistently led to a decrease in overshoot as well as in the rate of rise and rate of decay of the action potential The net result was a slight increase in duration of the action potential in the hypertonic medium

Eight fibres were stimulated with the frequency used for the active state recordings (quick release) *i e* 4 successive stimuli were given at intervals of about 150 ms The action potentials obtained in each respective fibre were superimposed on the same film frame during the photographic recording The results from one of these experiments are shown in Fig 5 As can be seen the action potentials recorded during the 4 different cycles were identical The same finding was obtained in the other 7 fibres studied

Discussion

The effects of hypertonic solutions on the mechanical activity of frog skeletal muscle were investigated in several previous studies (Overton 1902, Ernst 1926, Howarth 1958, Caputo 1968, Edman and Andersson 1968, Gordon and Godt 1970). It was demonstrated that hypertonicity of the extracellular medium reduces the tension output in response to a variety of stimuli: electrical stimulation, addition of caffeine or depolarization of the fibre in a high potassium medium (Gordon and Godt 1970). The cause of the reduction in tetanic tension was attributed (Edman and Andersson 1968, Gordon and Godt 1970) to a direct effect on the contractile proteins caused by an increased intracellular ionic strength. However, when discussing the reduction in twitch response it is necessary to consider not only a change in the maximum capacity of the contractile system to produce tension but also to take into account changes in the active state duration.

Howarth (1958), using frog sartorius muscle, found that in 1.5 R (0° C) the time to peak twitch tension was prolonged by 40—60 ms and that the active state decayed with a delay of the same amount. But he concluded that hypertonic solutions did not markedly affect the total duration of the active state.

In the present study the effects on the active state kinetics produced by changes in tonicity were analysed in single muscle fibres. The results showed that during a single twitch a shortening of the plateau phase of the active state is produced in 1.44—1.67 R. However, during repeated stimulation there is a prolongation of the plateau of the active state and also a slowing of the decay phase of the active state curve. The apparent discrepancy between the response to a single stimulus and to repetitive stimulation in the hypertonic medium can most probably be explained on the basis of changes in the calcium kinetics of the excitation contraction coupling. It is commonly accepted that the sarcoplasmic reticulum by release and re-uptake of calcium regulates the contractile activity in a skeletal muscle fibre (Sandow 1965, 1970, Ebashi and Endo 1968). It is reasonable to suppose that the active state reflects the concentration of calcium that is bound at any given moment to the contractile system (see Ebashi and Endo 1968, Edman 1970, Sandow 1970, Edman and Kiessling 1971). This concentration of calcium is a function of the amount of calcium released during excitation and of the rate of re-uptake of calcium by the sarcoplasmic reticulum. Evidence has been presented by Hodgkin and Briggs (1968) that by immersion of frog sartorius muscle in a hypertonic medium (2.2 R) the ability to release calcium with electrical stimulation is impaired. In barnacle muscle fibres hypertonicity caused a suppression of calcium release and of tension response as was demonstrated by Ashley and Ridgway (1970). The present findings on single fibres showing a shortening of the maximum intensity of the active state after a single stimulus are also consonant with the idea that there is a reduced release of calcium in hypertonic solution.

It is possible that the structural changes in the sarcoplasmic reticulum which are known to occur in hypertonic medium (Huxley, Page and Wilkie 1963, Freygang, Rapoport and Peachey 1967, Birks and Davey 1969) are associated with a reduced

ability to retain calcium within the reticulum Then the muscle cell will contain a reduced amount of calcium available for release This might explain the results of Homsher and Briggs (1968) referred to above and also the findings of the present investigation

If the ability to retain calcium within the sarcoplasmic reticulum is impaired it might be expected that hypertonic solutions will cause an initial release of calcium from the intracellular stores Indeed Isaacson (1969) demonstrated an increased efflux of calcium from frog sartorius in hypertonic medium and also an increase of tension by 1—3 per cent In single muscle fibres phasic contractures can be obtained in hypertonic solutions starting in 1 8 R and in 2 5 R reaching an amplitude of up to 40 per cent of the tetanus tension in normal Ringer s solution (Lannergren and Noth 1972) Furthermore hypertonic solutions with an osmolality of more than 2 R cause an increase in the heat production in frog s skeletal muscle (Yamada 1970) This was suggested to be due to a relase of calcium inside the cell thereby stimulating the oxidative phosphorylation (Yamada 1970) Taken together these findings are consistent with the idea that hypertonic solutions can cause a release of calcium in skeletal muscle

It might be argued that the step wise prolongation of the decay of the active state in hypertonic solution shown in the present investigation was due to a successive prolongation of the action potential duration but the results seem to rule out this possibility It was demonstrated that the action potential did not undergo any change in response to four successive stimuli given at intervals of 150 ms *i e* at the same intervals as in the quick release experiments

Maintained subthreshold depolarization of a muscle is known to be associated with a prolongation of the active state This effect was not related to an increase in the action potential area (Clinch 1968) In several previous studies hypertonic solutions were reported to cause a depolarization of the muscle (Tigyi and Shih Fang 1962 Gordon and Godt 1970) but the measurements were usually performed in solutions of very high tonicity 3—4 R. The present experiments did not show any marked changes of the membrane potential in 1 44—1 67 R In some fibres there was a slight depolarization but more often a hyperpolarization of 3—8 mV was seen These findings are in agreement with those reported by Yamada (1970) and seem to exclude depolarization as a cause of the active state changes seen in the present study

The prolongation of the plateau phase of the active state after repetitive stimulation and the step wise prolongation and slower rate of decay of the falling phase found in the present experiments suggest that the rate of calcium sequestration by the sarcoplasmic reticulum is decreased Supporting this explanation the experiments of Martonosi and Feretos (1964) and of de Meis (1968) showed that the ability of skeletal muscle microsomes to take up calcium in the presence of ATP was decreased when the KCl concentration of the medium was elevated

On the basis of the above considerations the following explanations of the experimental results would seem reasonable 1) The decrease in duration of the

active state during a single twitch observed in hypertonic solution is due to a diminished release of activator calcium 2) The slowing of the decay of the active state is due to a decreased rate of re uptake of calcium by the sarcoplasmic reticulum This is consistent with the finding that the duration of the active state is progressively increased by repeated stimulation in the hypertonic medium The impaired rate of removal of calcium will lead to accumulation of calcium in the myofibrillar space with each new stimulus This causes a prolongation of the active state as a longer time will be required to lower the calcium concentration to a given level

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Acta physiol scand 1973 88 160—174

From the Institute of Neurophysiology University of Oslo Norway

Unit Activity of Ganglionic and Medullary Second Order Neurones in the Eel Lateral Line System

By

EGIL ALNÆS

Received 24 July 1972

Abstract

ALNÆS E *Unit activity of ganglionic and medullary second order neurones in the eel lateral line system* Acta physiol scand 1973 *88* 160—174

Unitary spike activity has been investigated in the ganglion and the central terminational field of the R lat. post N X No dorsal root reflex is observed in the intracranial rootlet There is an excitatory coupling between ganglionic cells in the N lat post which can lead to multiple firing in these cells by stimulation of afferents in the trunk nerve Second order neurones were identified by their location and variable spike latency to peripheral nerve stimulation These cells are driven by a large number of active primary afferents from extended parts (order of magnitude 15 cm) of the trunk lateral line The statistics of spontaneous discharges were studied and compared with that of primary afferent nerve fibres About 1/3 of secondary neurones are either excited or inhibited from the contralateral lateral line nerve No evidence was found for inhibition from ipsilateral lateral line sensory organs

Introduction

No account has been published on the activity of higher order neurones in the mechanoreceptive lateral line system of teleosts despite much current research on its peripheral mechanisms (see Dijkgraaf 1963 Cahn 1967 Flock 1971 a b for references) Higher order neurones in the rhombencephalic lateral line lobe of electrosensitive *Gymnotidae* were investigated by Enger and Szabo (1965) Such units displayed complex firing patterns to moving electrical fields The influence of electrical fields on central neurones in the elasmobranch has also been reported in a preliminary study by Nicholson *et al* (1969) In neither case was it possible to elucidate the hierarchial order of these neurones and/or relate firing patterns to those of primary afferents or their central neuronal connections Recently a study of central nervous neurones in the mechano-sensitive lateral line system of the elasmobranch *Scyliorhinus canicula* (dogfish) has been advertised by Paul and Roberts (Roberts 1972) The spontaneous and evoked activity of primary lateral line afferents in the eel has been investigated by the author (Alnæs 1972 a b) Efferent inhibitory neurones are absent in this lateral line system (Alnæs 1972 b) and by field

potential analysis and EM study, the primary afferents have been shown to make extensive excitatory synaptic contacts with dendritic spines on neurones underlying the crista cerebellaris (Alnæs 1972 c) The eel lateral line lobe thus seems a sufficiently simple structure to permit a study of well defined second order neurones in a teleostean lateral line afferent pathway

Methods

For experimental procedure see Alnæs (1972 a, b c) Intracellular recordings from ganglionic cells were obtained through 2 M KCl microelectrodes of 10—25 MΩ resistance Transmembrane current pulses were delivered through a bridge device (M4A WI Instruments Inc) Extracellular action potentials were recorded by NaCl microelectrodes of 5—10 MΩ resistance at a depth of 250—500 μm from the surface of the crista cerebellaris and recorded on magnetic tape Firing patterns were analysed on line and off line by a Nord 1 digital computer A comprehensive programming system was developed for simultaneous monitoring of analogue and digital parametres of nervous activity

Results

I Interaction between neurones in the ganglion

As mentioned in Alnæs (1972 a) single electric stimulation pulses of the trunk lateral line nerve would sometimes (approximately 10 % of units) give rise to double or triple spike responses in ganglionic primary afferents However no evidence was found for peripheral unidirectional synapses in this nerve (Alnæs 1972 a) The additional spikes might conceivably arise from a long lasting depolarization of axonal terminals analogous to a dorsal root reflex presynaptic inhibitory processes possibly playing a part in cristal activity

In the R lat post N X of the eel rounded cell somata of up to 30 μm diametre are found to envelop the nerve prior to its joining the medulla oblongata (Alnæs unpublished) The cells are pseudounipolar with a rather thick initial segment Their continuity with nerve axons is easily verified in Bodian sections However no numerical analysis has been performed and it is possible that not all primary afferent somata are situated within this ganglion In the EM the cell bodies are seen to be myelinated one or more Schwann cells tightly enveloping the soma leaving however a naked axon hillock Between this region and the myelinated large calibre parts of the axon a pronounced axonal narrowing was found in all cells Multiple dense strands of packed microfilaments are scattered throughout the cytoplasm prominently visible also in the Bodian stained sections

The surface potential recorded from the intracranial portion of the N lat post (Fig 1) consists essentially of a triphasic response (for cut end neurogram see Fig 2 Alnæs 1972 a) A small positivity is followed by a sharp negative inflection caused by activity in the thick axonal group (Alnæs 1972 c) This spike component is succeeded by a slow positive wave The positive wave decreases from the ganglionic part of the rootlet towards the medulla (Alnæs 1972 c Fig 1 B) It is therefore not an electrotonically conducted dorsal root potential transmitted from depolarized

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was often of larger amplitude than spontaneous or evoked action potentials in the same cells whereas the opposite did never occur Thus a conduction block from axon to soma is suggested the slow depolarization facilitating spike invasion towards the electrode (soma) Late spikes always occurred with delays exceeding 6 ms compared with the rapidly conducted axonal spikes Fig 1 C shows this late spike to be sensitive to repetitive stimulation whereas the axon conducts spikes well above 100 c/s (Alnæs 1972 a) and the slow depolarization is but little reduced Hyper polarizing of the cell bodies by transmembrane current injection (Fig 1 C lower records) abolished the somatic spike generation Depolarizing currents (Fig 1 D middle record) produced a spike similar to those that were triggered by the late potential In this neurone the orthodromic axonal spike did in fact invade the soma (upper record) whereas this invasion did not occur when the cell was hyper polarized (lower record) In this instance the diphasic rising phase of the invading action potential is clearly displayed The amplitude of the axonal spike after somatic hyperpolarization corresponded to the inflection of the somatic spike whether this was DC induced or triggered by the late potential (note arrows) This suggests that the blocking of the axon spike and the triggering of the spike by the slow somatic depolarization take place at the same site peripheral to the recording site

In a few neurones late spikes occurred at latencies and intensities compatible with excitation from slowly conducting (< 14 m/s) axons in the ganglion

Cutting of the N lat post centrally to the ganglion caused deterioration of ganglionic cells as measured by their membrane potentials However in one experi ment double spike firing was recorded even under such conditions Thus it was con cluded that some excitatory synaptic coupling exists between afferents in this ganglion The amplitude of the coupling potential was not measurably influenced by hyperpolarization of the membrane potential (Fig 1 C) This and the high fol lowing frequency suggest that the excitatory coupling between the ganglionic cells is electrical The coupling potentials generate the slow positive wave which can be recorded extracellularly at the site of cell somata and neither this wave nor the extra spike firing can be attributed to a dorsal reflex response It is emphasized that pontaneously occurring spikes are not followed by slow potentials and/or synaptical ly induced spikes as witnessed by Fig 1 B and the unimodal spike interval histograms observed for all primary afferents (Alnæs 1972 a) Nor were spontaneously occurring slow potentials observed

II Single unit identification in the subcristal region

The subcristal region defined through earlier field potential and histological analysis (Alnæs 1972 c) was explored by conventional extracellular microelectrode technique during peripheral *en passage* stimulation of the posterior lateral line nerves Evoked or spontaneous action potentials of 0 2—4 mV amplitude were recorded at a depth of 250—500 μm from the cristal surface This corresponds to the location of the sub cristal cell bodies In this region recordings were made of three distinct classes of single units

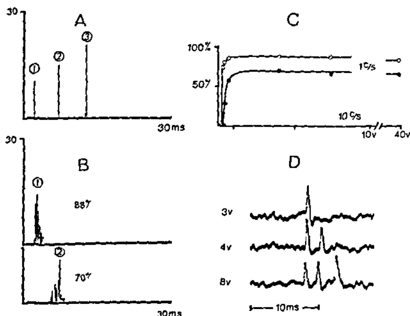


Fig 2 A Post stimulation histograms of spike responses of primary afferent fibre in the subcristal area Three peripheral nerve *en passage* stimulation sites Bin width 0 3 ms B Post stimulation histograms of spike response of subcristal second order neurone 50 stimulations at two different peripheral electrodes Response percentage indicated C Response percentage of unit shown in B as function of stimulation intensity and frequency Open circles proximal stimulation site Closed circles distal stimulation site Triangle proximal stimulation at 10 c/s D Multiple spike response of subcristal neurone recruited by increasing nerve stimulation intensity

(1) A number of units were identified as primary afferents displaying all characteristics established for such neurones during a study of the lateral line nerve (Alnæs 1972 a) *ti* size of peripheral receptive fields threshold/conduction velocity ratios statistics of spontaneous discharges *etc* In Fig 2 A the constant latency typical of primary afferent fibres is displayed in an on line computer histogram of 0 3 ms bin width This unit was not spontaneously active and had its receptive field located in the tail region In this experiment it was stimulated *en passage* at 3 different sites along the peripheral nerve The spike responses fell mainly in a single 0 3 ms bin *also for the few slowly conducting afferents picked up in this study* Primary afferents were found throughout the entire subcristal region confirming earlier histological and functional interpretations (see Alnæs 1972 c for commentaries) as well as below the lateral surface curvature of the medulla in the rostral direction Primary afferents are seen to form a rostral tract to the cerebellum in the corresponding part of the medulla

(2) A number of spontaneously discharging subcristal units were also found where peripheral nerve stimulation did not produce any changes in the discharge pattern as analysed and displayed on line by the computer Such units were not included in the present investigation

(3) 68 subcristal neurones in this study responded to peripheral nerve stimulation by one or more action potentials at latencies that were more variable than those described for primary afferent terminals A typical poststimulus histogram is presented in Fig 2 B Latency variations commonly ranged from 1 2—3 0 ms with a mode at 2 ms No units were found that showed intermediate responses between this pattern and that of primary afferents and thus this served as an identification criterion All units were encountered below the molecular layer (as identified by the field potential reversal) at penetration depths 250—500 μm (mode 300 μm) within the zone of pronounced surface field potentials (Fig 4 in this paper and Fig 1 Alnæs 1972 c) However no units were isolated in vertical penetrations of the medial 20 % of the crista cerebellaris where the molecular layer forms the medial lip of the cristal ridge Thus the spatial distribution of these units conforms to the one established for the second order lateral line lobe neurones (Alnæs 1972 c) The initial evoked spikes coincided in time with the P_1—P notch in the depth field potential (Alnæs 1972 c) and all responses studied occurred within the period of P response depth negativity (Alnæs 1972 c) Usually less than 100 per cent of suprathreshold nerve stimulations gave rise to an evoked spike and this percentage might vary along the nerve (Fig 2 B and C) This will be commented upon later

The unitary response was seen to be frequency sensitive (Fig 2 C 10 c/s) Most units were recruited within the threshold range of large calibre afferents (Fig 2 C) and some units responded with additional spikes on increased nerve stimulation (Fig 2 D) The spike response corresponds in time with the EPSPs recorded intracellularly and it is concluded that this group of single units represents subcristal secondary lateral line neurones (Alnæs 1972 c)

III Statistical description of the spontaneous activity of secondary neurones

Statistical discharge analysis was carried out in the same way as described for primary lateral line afferents (Alnæs 1972 a) The frequency of spontaneous discharge in secondary neurones ranged from 50 c/s to zero as is the case with primary afferents in the same experimental condition (Alnæs 1972 a) Of 68 units 9 (13 %) were not spontaneously active The percentage of silent primary afferents under the same conditions (immobilized and extended fish in air) is known to be about 25 % (Alnæs 1972 a) All interval histograms of spontaneously active units were unimodal (e g Fig 3 E) as was also found for peripheral receptor activity in the immobilized fish (Alnæs 1972 a) However whereas sensory organ discharges in the proximal lateral line region were often influenced by gill movements (Alnæs 1972 a) no such coupling of activity was discernible in the central units Since proximal lateral line afferents innervate cells in this group it must mean either that their influence is slight or that some smoothing of input activity takes place The distribution of mean interval ($\bar{x}$) computed for 38 units where statistical analysis was satisfactory (see Alnæs 1972 a for criteria) is plotted in Fig 3 A The histogram shows a mode at $x \approx 50$ ms and a mean $\bar{x}$ of 81 ms was computed the corresponding values found for large calibre primary afferents (Alnæs 1972 b) were 70 ms and approximately 200 ms

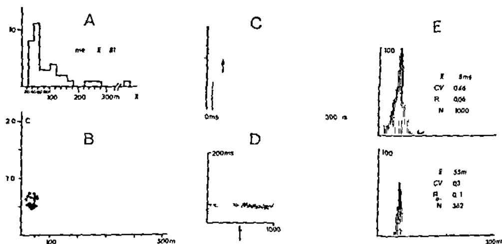


Fig 3 A Distribution of mean spontaneous firing interval ($\bar{x}$) for 38 subcristal second order neurones B Scatter plot of units in A Abscissa $\bar{x}$ Ordinate Coefficient of variation (CV) C Computer spike histogram of spontaneously active second order neurone Successive sweeps from above downward Posterior lateral line nerve cut proximally at time of arrow Later nerve stimulation still elicits medullary spike response D Time histogram of spontaneously active unit. Successive intervals (ordinate) plotted by succession number along the abscissa. Arrow Nerve cut 15 cm distally E Interval histograms of unit in D before (upper) and after nerve transection (lower histogram) Discharge statistics are indicated as computed

Serial correlation coefficients for neighbouring intervals (R_{0-1}) were distributed over a wider range than found for primary afferents (Alnæs 1972 a) In the present series of experiments values from −0 19 to +0 34 were computed (mean R_{0-1} = 0 07) The regularity of firing as measured by the coefficient of variation (CV = SD/$\bar{x}$ was significantly lower than that observed for primary afferents Fig 3 B is a scatter plot where CV (ordinate) is plotted against $\bar{x}$ (abscissa) for units having $\bar{x}$ < 500 ms This figure should be compared with Fig 1 C and D of Alnæs (1972 b) As can be seen the central units displayed a more heterogenous CV/$\bar{x}$ interrelationship than do the primary afferents Also no evidence for discrete subgroups can be discerned No units displayed CV < 0 35 the distribution showing a mode at 0 55 The corresponding mode for primary afferents is found at CV = 0 25 (Alnæs 1972 a)

Thus the population of second order neurones investigated displayed the following dissimilarities from the population of primary afferents feeding into the area

(1) a lower percentage of silent units
(2) absence of regular units (CV < 0 35) and a higher mean value of irregularity
(3) a more heterogenous CV/$\bar{x}$ interrelationship with no evidence for subgroups
(4) no activity changes coupled to gill movements
(5) a tendency to a higher mean rate of firing (within the same total range)
(6) a wider range of inter interval dependency (measured as R_{0-1}) but with

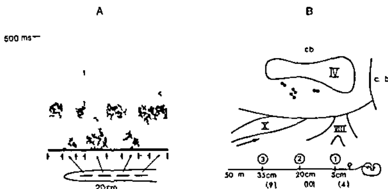


Fig 4 A Time histogram of spontaneously active subcristal secondary neurone Each dot corresponds to one spike height above baseline indicates time interval to preceding spike The experiment proceeded from left to right along the abscissa (spike succession number) Arrows indicate periods where neighbouring 5 cm portions of the lateral line area were gently brushed Stippled line indicates periods without stimulation. B Surface location of penetration tracks (cr cb crista cerebellaris c cb corpus cerebelli) Symbols indicate which of 3 peripheral nerve electrodes was the most distal in evoking spike response in 23 subcristal neurones

corresponding means (Primary afferents +0 06 and secondary afferents +0 07) Possible explanations of these differences in firing may be

(a) that the central units receive tonic input also from other sources than posterior lateral line receptors

(b) that a rather large number of disparate primary afferent input channels are simultaneously integrated by the central neurones

Experiments in which the activity changes induced by nerve transection were studied gave support for (b) and suggested that the background activity of the secondary neurones is mainly generated by primary afferent activity from the lateral line nerve Fig 3 C shows a computer generated poststimulus dot histogram Each dot corrsponds to a spike within a 300 ms window from the nerve stimulus event and the plot should be read in successive sweeps from top to bottom The upper third of the plot corresponds to spontaneous activity (0V stimulus) At the time of the arrow the posterior lateral line nerve was transected at the level of the operculum All spontaneous activity disappeared In the lower third of the plot nerve stimulation proximal to the transection site gave rise to a latency variable spike response in the unit demonstrating that it had not been lost Identical proximal transection experiments on three active units located within the maximal lateral line synaptic field all gave similar results

Fig 3 D and E show the influence on unit activity by nerve transection at a more peripheral level of the posterior lateral line nerve D is a time histogram Successive intervals (ordinate) are plotted by their number along the abscissa (see Mires 1972 a for similar plots) At the time of the arrow the nerve was transected 15 cm distally The interval pattern was immediately changed This is brought out more quantitatively in the interval histograms of E showing the situation before and after transection respectively It is seen that the unit became more regular (lower CV)

and that short intervals (less than the mode value) essentially disappeared Similar effects were obtained in a number of other experiments Confirming the conclusion reached above this demonstrates in addition that the secondary neurones are influenced by peripheral sensory organs at disparate locations along the lateral line canal

IV Receptive fields of secondary neurones

Mechanical punctate stimulation along the lateral line canal was insufficient to determine the peripheral receptive fields of most secondary neurones Usually only diffuse and inconsistent discharge changes were brought about by such stimulation which is known to excite peripheral sensory organs (Alnæs 1972 a) Obviously the activity changes originating from single or scattered sensory organs are smoothed by the integrative processing in the secondary neurones

Light mechanical stroking along the lateral line canal area was often a more effective way of stimulation In the experiment illustrated in Fig 4 A neighbouring 5 cm portions of the fish were gently brushed by a wetted piece of tissue paper The spike activity was increased above its mean spontaneous rate within a field measuring approximately 20 cm in the distal region of the fish Primary afferent units are known to respond to similar stimulation only within fields overlying a single canal organ (Alnæs 1972 a)

The mean order of magnitude of peripheral fields was assessed also by a more indirect method The peripheral nerve was stimulated *en passage* (see Methods Alnæs 1972 a) at three different sites dividing the lateral line canal area in three equally sized fields of approximately 15 cm length In successive experiments the most distal electrode site from where a given secondary neurone could be influenced was noted Fig 4 B shows the central location of the 23 cells identified in these experiments No somatotopic pattern was observed within the lateral line lobe

If the peripheral field supplying any given secondary cell were small (compared with 15 cm) each of the three electrodes would be expected to be the most distal influencing site for equal number of cells On the other hand if each secondary cell samples sensory activity from the whole length of the fish (peripheral field $\gg$ 15 cm) the tail electrode would influence all cells If the peripheral fields have a mean order of magnitude $\approx$ 15 cm the two posterior electrodes would influence equal numbers whereas the opercular electrode (having few proximal sensory organs in front of itself) would be the most distal electrode for fewer units The latter situation is found to be the case

Approximately 1/3 of the secondary neurones investigated were also influenced by *en passage* electrical stimulation of the contralateral nerve 5—10 cm distally to the operculum However no attempt was made to assess the extension of contralateral peripheral sensitive fields 3/5 of these units showed responses similar to those evoked by ipsilateral nerve stimulation one or more action potentials being recruited at increasing stimulation intensities The unit shown in Fig 5 A was excited by bilateral nerve stimulation at identical peripheral levels of the two nerves The latency com

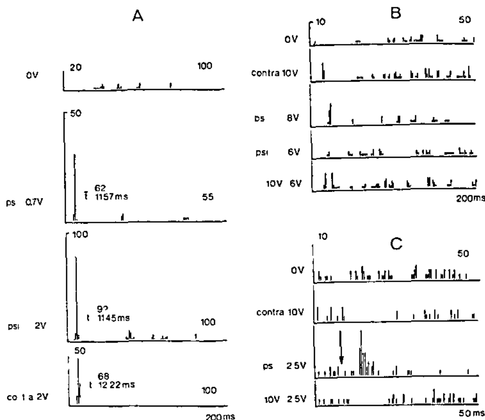


Fig 5 A—C Post stimulation histograms of spike activity in 3 second order neurones A Stimulus intensity and laterality respon e percentage latency (computed on l ne) and number of nerve stimulations are indicated B Summation of supra threshold contralateral and sub threshold ipsilateral nerve stimulation (visible in lower record at their respe tive latencies) C Inhibitory effect of contralateral nerve stimulation Spontaneous (second record) and evoked spikes (lower record) are suppressed Arrow marks timing of ipsilateral nerve stimulation

puted for the contralaterally evoked response was 0 8 ms longer than that computed for ipsilateral stimulation Lags of similar magnitude were computed in those units where excitatory responses were induced by low threshold primary afferents in both nerves This delay corresponds to the conduction time of crossing primary afferents (Alnæs 1972 c)

The excitatory influence from the two sides was most often found to be different as measured by spike recruitment/stimulation intensity plots like those shown in Fig 2 and 4 Contralateral excitation might be mediated mainly through either large or small calibre primary afferents respectively and such predominance was not correlated with that found for ipsilateral nerve stimulation Usually contralateral s imulation induced smaller responses (as measured by evoked spikes per stimulus) than ipsilateral stimulation although in occasional units the opposite was found A

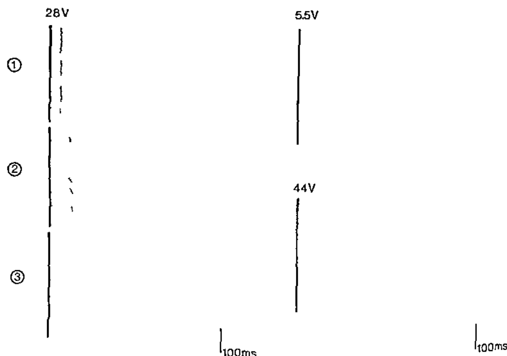


Fig 6 A Post stimulation histograms as in Fig 3 C of one spontaneously active second order neurone Supramaximal nerve stimulation at three peripheral *en passage* nerve stimulation sites site (1) being most proximal 50 sweeps B Another unit stimulated at submaximal and supramaximal nerve stimulation intensities from one electrode site Further explanation in text.

summing effect of nearly coincident suprathreshold contralateral and subthreshold ipsilateral stimulation is shown in Fig 5 B

2/5 of those units that were influenced by the contralateral nerve were inhibited by such stimulation (Fig 5 C) The spontaneous activity ceased for a period $\geqslant 10$ ms without any preceding excitatory response The onset of this silent period commonly occurred approximately 2 ms after the arrival of large fibre primary afferent input to the area Contralateral inhibition was found to be mediated both by thick and thin fibre groups respectively (as measured by thresholds) in the present material Fig 5 C shows a summing effect of the two opposite lateral line influences in one such unit

No evidence was found for similar inhibition in the ipsilateral input to the lateral line lobe in the present series of nerve stimulation experiments even when the nerve was stimulated distally to the most peripheral electrode yielding an excitatory response It might be argued that secondary neurones receive combined excitatory and inhibitory influence from ipsilateral nerve stimulation The method of *en passage* nerve stimulation at discrete nerve sites is not ideally suited to investigate this possibility Proximal nerve stimulation elicits a mixed nerve excitation as well as anti

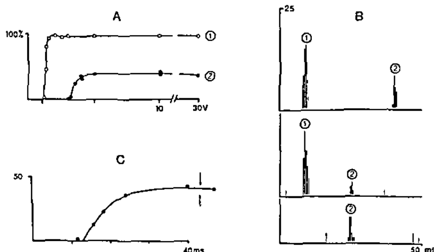


Fig 7 A Different response percentage stimulation intensity relationship for one unit at proximal (1) and distal (2) nerve electrodes B Post stimulation histograms of unit in A at suprathreshold stimulations from electrodes 1 and 2 At reduced stimulus interval (middle record) stimulation at electrode 2 elicits fewer spikes in secondary neurone (compare with controls in upper and lower records) Each histogram consists of 50 stimulus sweeps Bin width 0 5 ms C Distally evoked response plotted as function of interval to preceding proximally evoked response (from records similar to those shown in B) Arrows indicate variation in distal response when evoked in isolation

dromic invasion of the pertinent sensory organs (Alnæs 1972 b) and any inhibitory influence from the rostral sensitive field would be partly camouflaged The complexity of the experimental analysis can be assessed from plots like Fig 6 A and B Stimulation at a proximal electrode site induced a spike response which is usually (Fig 6) but not always followed by a pause in spontaneous activity This pause cannot readily be ascribed to inhibitory synaptic activity since it would also be influenced by the refractoriness of the central neurone as well as the antidromic blotting out of orthodromic activity (Alnæs 1972 b) This pause was often (but not always) seen to be shorter when elicited at more caudal *en passage* stimulation sites (Fig 6 A) and often (but not always) to be prolonged at increased stimulation intensities (Fig 6 B) Neither finding reduces the possibilities Transection of the nerve behind the proximal electrode cuts off the background activity and cannot be used to resolve the problem The possibility remains that a concomitant inhibitory afferent volley is set up by *en passage* nerve stimulation but if so it did never override the excitatory effect as was observed for contralateral stimulation Distal sensory organ influence in the ipsilateral lateral line was always similar (although somewhat attenuated) to that of proximal influence (Figs 2 and 7) Excitation through small calibre afferent fibres (Alnæs 1972 a) was sometimes seen to be predominant either in rostral or caudal (Fig 7) ends of sensitive fields *In units of the latter type selective excitation* of the two sets of sensory organs influencing the subcristal neurone was possible

Fig 7 A shows the evoked response/stimulation intensity plots computed for the proximal and distal stimulation electrodes From the threshold and latency data the distal electrode was shown to elicit responses via thin primary afferent fibres conducting < 14 m/s, whereas the proximal electrode recruited a near 100 % response through excitation of thick afferent fibres conducting at approximately 25 m/s Fig 7 B and C show that the distal response was reduced if it was elicited < 20 ms after a proximally induced secondary spike This period probably corresponds to the refractory period of the second order neurones (Alnæs 1972 c) and does not support the notion of a prolonged inhibitory synaptic process

Discussion

The present investigation demonstrates that some excitatory coupling exists between primary afferent cell somata in the extramedullary ganglion of the posterior lateral line nerve The observed independence between synaptic potentials and spike activity of a given ganglionic neurone shows the effect not to be caused by delayed somatal invasion and/or private axonal collaterals The neuronal coupling is suggested to be electrotonic since the depolarizing potential is not appreciably changed by hyperpolarization of the cell

The erratic and easily blocked IS S invasion observed for orthodromic (somatopetal) spikes is probably due to the narrow unmyelinated axonal initial segment observed electronmicroscopically

Electrotonic junctions have been demonstrated between spinal neurones in teleosts (Bennett *et al* 1963) and between medullary neurones in various fishes (Bennett *et al* 1967 I—IV) Recently Baker and Llinas (1971) have reported electrotonic coupling between neuronal somata in the mesencephalic trigeminal nucleus of the rat It is still conjectural what role if any such coupling plays in the normal impulse traffic in the lateral line nerve since fully adequate stimulation procedures have not been worked out for this sensory system However in the experimental situation where synchronous action potential volleys are set up by electrical nerve stimulation the relative importance of this phenomenon is probably enhanced Possibly the recruiting of several consecutive spikes (Fig 2 D) observed in subcristal neurones upon increasing nerve stimulation intensity may partly be due to such ganglionic spike recruitment The morphological correlate of this functional contact has not been sought in this investigation and it remains for future work to study the topographical lay-out in terms of peripheral fields of the contacting cells

There is good agreement between the interpretations reached on neuronal activity in the crista region from field potential and histological analysis (Alnæs 1972 c) and the results reported in this paper Excitatory effects are caused by converging monosynaptic input on subcristal neurones from both lateral line nerves and summation in individual cells has been observed The inhibitory influence from contralateral sensory input was not suggested by the field analysis of the previous paper (Alnæs 1972 c) However if sizable contingents of contralateral primary afferents

induce approximately synchronous excitatory and inhibitory synaptic activity on the subcristal neuronal population this would possibly partly explain the attenuated field response observed upon contralateral nerve stimulation The subcristal dendritic arborizations receive pleomorphous presynaptic contacts (Alnæs 1972 c) but naturally the morphological correlate or origin of the inhibitory synaptic activity remains to be elucidated Obvious possibilities would be inhibitory activity mediated through axons (-collaterals) of the secondary cells either from the ipsilateral or (in directly) from the contralateral side

The essence of the present investigation is the large degree of excitatory influence by lateral line afferent nerve fibres on subcristal neurones The size of secondary cell sensitive fields seems surprising in view of the discrete and punctate organization of sensory organ contribution to the lateral line nerve The lateral line lobe is not the only relay station for afferent input however (Alnæs 1972 c) and possibly a somatotopic coding may play a larger part in more rostral nuclei But the subcristal area would be the first central area to receive lateral line information and the seemingly large diffusion of the spatial information in the lateral line afferents is puzzling The integrative interplay between lateral line and audio vestibular input has not been studied in this context but remains a functionally important factor as judged by the histology (Alnæs 1972 c)

In preliminary studies with freely submerged fishes stroking by blunted glass rods along the lateral line in one direction was sometimes found to be a more efficient way of exciting a given subcristal neurone than stroking in the opposite direction (Alnæs unpublished) If central neurones were connected to primary afferents of a given directional polarity (Flock 1965) only complex functions of this type might be performed by secondary neurones even in the absence of detectable in hibitory mechanisms However better methods for selective sensory organ excitation will have to be worked out to confirm this hypothesis Higher order neurones in electrically sensitive fishes have been shown to respond to rather complex changes in surrounding electrical fields (Enger and Szabo 1965)

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Differences in the Autoradiographic Localization of Labelled Morphine-Like Analgesics in the Mouse

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Abstract

Appelgren L E and L Terenius *Differences in the autoradiographic localization of labelled morphine like analgesics in the mouse* Acta physiol scand 1973 *88* 175—182

The distribution of the ^3H labelled narcotic analgesics dihydromorphine and fentanyl and the narcotic antagonist diprenorphine was studied by whole body autoradiography in male and female (pregnant) mice Very small amounts of label were found in the CNS after administration of dihydromorphine which was rapidly excreted via the urine There was very little passage of labelled material over the placental barrier Pretreatment with nonlabelled diprenorphine did not change the distribution After the administration of fentanyl a very different picture was seen Large amounts of label passed into the CNS In addition to label in urine large amounts were seen in the gall bladder and in the intestinal contents Only little passage over the placental barrier was observed After the administration of diprenorphine rather little label was found in the CNS while high amounts were excreted via urinary pathways and still more via the intestine There was considerable passage over the placental barrier

Morphine like activity is produced by a number of amines with very different structures Much work has been devoted to studies on relationships between structure and activity (Beckett and Casy 1962 Porthogese 1966 Janssen and van der Eycken 1968) A variety of testing procedures have been used generally based on parenteral administration Since several of the morphine like drugs markedly differ in physico-chemical properties a variety of pharmacokinetic factors will influence the outcome of such experiments

In recent contributions Herz and co-workers (see Herz and Teschemacher 1971) study analgesic effects of various drugs following local *i e* intraventricular application These authors found quite different estimates of potency after the local application compared with the conventional tests showing that pharmacokinetic factors add to the complexity of structure activity studies

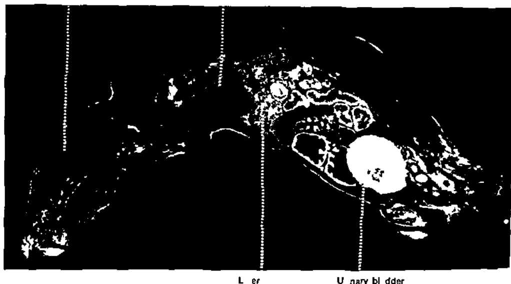


Fig 1 Whole body autoradiogram showing the distribution of dihydromorphine ^3H min after i v injection into a male mouse (White areas correspond to high amounts of radioactivity) Very rapid excretion of radioactivity is apparent from the high concentration of ^3H in the urinary bladder The central nervous system has no registrable radioactivity

The present work compares by the use of autoradiography the whole body distribution of three compounds representing different classes of morphine like analgesics dihydromorphine which is biologically very similar to morphine (Eddy *et al* 1956) diprenorphine which is a compound in the extremely potent oripavine series (Lewis *et al* 1971) and fentanyl (Janssen *et al* 1963) a highly potent and fast acting analgesic the chemical structure of which bears no resemblance to the morphine alkaloids It was found that the distribution of these drugs after intravenous administration was markedly different

Materials and Methods

11 mice of the N M R I strain either adult males eighing 19—20 g or pregnant females of about 40 g b wt were used The test substances ere dissolved in isotonic saline All injections were done intravenously into the tail vein in conscious animals The injection time was about 30 s The experiment was terminated by immersing the animals in hexane/solid carbon dioxide (−78 C) *after a short period of ether anesthesia* The mice were embedded in a mixture of carboxymethyl cellulose and water Sagittal sections (20 μm) of the body were cut and dried at −10 C according to the Ullberg autoradiographic technique by which each section is attached onto tape (No 810 3M Co) (Ullberg 1954) The tape mounted sections were pressed against G5 nuclear emulsion plates (Ilford) The exposure time varied from 1 to 8 months Developing of the plates was done after removal of the tape mounted sections and some of the sections were stained with hematoxylin and eosin

The following labelled analgesics were used dihydromorphine 7 8 ^3H prepared by catalytic reduction of morphine with tritium specific activity 24 μCi/μg fentanyl ^3H generally labelled in the phenyl ring specific activity 0 3 μCi/μg and diprenorphine (M 5050) ^3H labelled in the 8 position specific activity 0 3 μCi/μg The dihydromorphine was repurified by thin layer

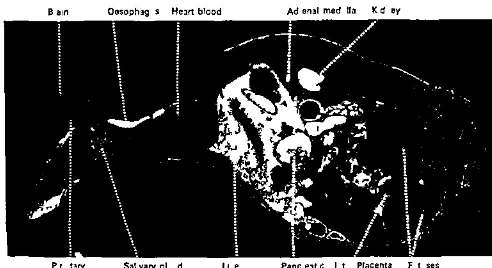


Fig 2 Whole body autoradiogram showing the distribution of dihydromorphine ^3H 20 min after iv injection into a pregnant mouse Note the radioactivity in the adrenal medulla and pituitary The fetu es show very small amounts of ^3H

chromatography pr or to use fentanyl was about 95 % pure on thin layer chromatography while diprenorphine was used shortly after the supply from the manufacturer (Re kitt Ltd Hull England) who stated it to be about 98 pure

The dose given of dihydromorphine ^3H was 10 μg (corresponding to 240 μCi) and of fentanyl ^3H 1000 μg (1 mouse) and 500 μg (2 mice) (corre ponding to 300 μCi and 150 μCi respectively) and of diprenorphine ^3H 800 μg (corre pond ng to 240 μCi) The survival t mes for the mice injected dihydromorphine ^3H were 5 20 and 120 min for the mice injected with fentanyl ^3H 1 5 5 20 and 60 min and for the mice injected with diprenorphine ^3H 20 and 60 min One male mouse with a survival time of 20 min wa gi en 2 μg non radioact ve diprenorphine immediately before the injection of dihydromorphine-^3H

Results

Dihydromorphine ^3H As soon as 5 min after injection of dihydromorphine ^3H there was a remarkably high concentration of tritium in the urinary bladder indicating a very rapid excretion (Fig 1) Also after 20 and 120 min high levels were found in the urinary excretion organs In the oesophagus and the gall bladder high amounts of tritium were also found but the concentration in the intestinal contents was rather low

The levels of radioactivity in the central nervous system were very low in comparison with most other tissues The plexus choroideus which is known to transport morphine like analgesics (Hug 1971) was quite distinctively labelled as was the pituitary gland and the adrenal medulla Rather high amounts of tritium were present in the retina and in the peripheral parts of the lens a slight uptake was registered Small amounts of radioactivity were also found in the pancreatic islets After 120 minutes radioactivity was still found in the salivary glands the adrenal medulla and the intervertebral discs in a concentration exceeding that of the liver

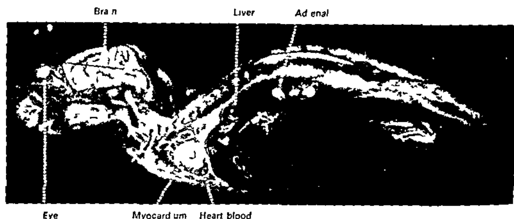


Fig 3 Whole body autoradiogram showing the distribution of fentanyl H 1 5 min after iv injection into a male mouse The myocardium adrenal and central nervous system contain high amounts of radioactivity

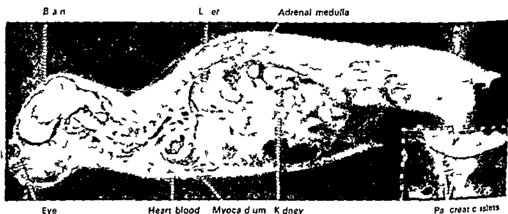


Fig 4 Whole body autoradiogram showing the distribution of fentanyl ^3H 5 min after iv injection into a male mouse Inserted detail of an autoradiogram from the same mou e showing the uptake of ^3H in the pancreatic islets

Very small amounts of radioactivity were found in the fetuses The distribution within the fetuses was even with the exception of the brain which was entirely void of radioactivity

Pretreatment with non radioactive diprenorphine did not change the di tribution of dihydromorphine ^3H and/or its labelled metabolites 20 min after injection

Fentanyl ^3H The higher dose of fentanyl ^3H (1000 μg) was almost lethal One male animal which survived for about 1 5 min was studied Two further animal which received 500 μg were studied 5 and 20 min after the injection One pregnant animal was studied 60 min after injection of the same dose It was found that the

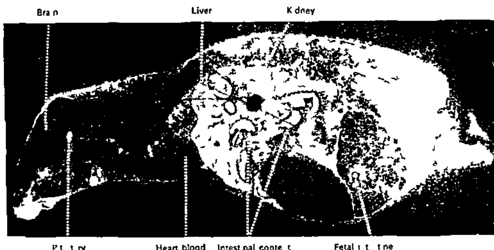


Fig 5 Whole body autoradiogram sl o ing the distribution of diprenorphine ³H 20 min after i v injection into a pregnant mouse Note the faint uptake of ³H in the brain and the relatively strong accumulation of rad oactivity in the fetal intestines

overall distribution of radioactivity markedly differed from that seen after injection of dihydromorphine ³H The central nervous system was heavily labelled and the grey matter contained more tritium than the white matter (Fig 3) Twenty min after the injection the frontal lobe hippocampus and the pituitary gland showed very high amounts of radioactivity In the peripheral ganglia the concentration of tritium was somewhat higher than in the peripheral nerves The plexus choroideus showed the same concentration of tritium as was registered in the grey matter of the brain

The lungs and the myocardium were strongly labelled a short time after injection After 20 and 60 min there seemed to be relatively more radioactivity in the gall bladder and the intestinal contents than in the urinary excretion organs in comparison with dihydromorphine The salivary glands showed rather strong radioactivity 20 min after injection In the islets of Langerhans the adrenal medulla and retina a marked accumulation was observed (Fig 4)

In the pregnant female slight accumulation of radioactivity was observed in the mammary glands The foetuses contained only small amounts of label

Diprenorphine ³H The distribution of labelled diprenorphine was studied in a male mouse 1 h after the injection and in a pregnant mouse 20 min after the injection The overall distribution picture was intermediate between those seen after dihydromorphine and fentanyl injections High concentrations of radioactivity were found in the urinary excretory pathways and still higher in the bile ducts and intestinal contents In the central nervous system there was a weak accumulation of tritium while the pituitary showed a marked accumulation Rather strong uptake was registered in the lens 60 min after injection

In the pregnant animal there was distinct evidence for passage of radioactivity over the placental barrier, since the fetal intestine was strongly labelled (Fig 5) In the mammary gland also some radioactivity was registered

The cartilage (in the intervertebral discs) was heavily labelled 60 min after injection

Discussion

The starting point for this investigation was the studies by Herz and co workers (Albus *et al* 1970 von Cube *et al* 1970 Kutter *et al* 1970) who showed that potency estimates in local (= intraventricular) applications of analgesics were markedly different from such estimates after peripheral injections It was therefore decided to study the influence of distributional factors on the availability to the central nervous system of some representative drugs by using whole body autoradiography Dihydromorphine which is structurally and biologically very similar to morphine (Eddy *et al* 1956 Mellet and Woods 1963) and fentanyl which has a very different structure (Janssen *et al* 1963) were both used by von Cube and others (1970) They also studied etorphine which was not possible for us because of its high toxicity and the limit posed by the available specific radioactivity Instead the structurally related diprenorphine which is a very potent morphine antagonist (Blane and Dugdall 1968) was used The dose of dihydromorphine used is quite within the range used for analgesia (Mellett and Woods 1963) while fentanyl had to be given in almost lethal doses (analgesic ED_{50} in mice about 10 μg Janssen and van der Eycken 1968) The doses of diprenorphine are also very high since it is extremely potent as an antagonist (ED_{50} about 16 μg/kg in mice Blane and Dugdall 1968)

Some whole body autoradiographic work also with mice and tritium labelled dihydromorphine has been published previously Because of the low technical quality of the pictures as shown they unfortunately do not allow much comparison with the present results Furthermore Hug (1963) used a higher dose of a preparation of lower specific activity (400 μg and 0 75 μCi/μg compared with our 20 μg and 24 μCi/μg respectively) As in our work the most marked accumulation was seen in the kidney and the digestive tract while the central nervous system was essentially negative Also a higher accumulation was seen in the adrenal medulla than in the cortex Autoradiography of C^{14} labelled morphine (800 μg per animal 2 7 μCi) has also been reported again showing accumulation in excretory pathways and in the adrenal medulla (Matsumota and Takahashi 1967)

A comparison between the whole body distribution patterns of the studied drugs and/or their metabolites reveals very marked differences While dihydromorphine was almost excluded from the central nervous system (Fig 1 and 2) diprenorphine was less so (Fig 5) and fentanyl was taken up very markedly (Fig 3 and 4) In other words a blood brain barrier is very active in excluding dihydromorphine from the central nervous system while fentanyl passes through without any difficulty In the pituitary gland the barrier is not operating resulting in an accumulation of all studied drugs and most markedly of fentanyl It is also apparent that the elimination

of dihydromorphine via the kidneys is extremely rapid resulting in low circulating levels For fentanyl and probably diprenorphine excretion via the intestine is also important

There were less pronounced differences in permeability over the placental barrier After dihydromorphine administration very little label was found in the fetuses while at least the fetal intestines were labelled after diprenorphine administration Interestingly the fetal brains were almost negative after fentanyl injection although the maternal brain was heavily labelled Obviously there is not necessarily parallelism between permeability over the blood brain barrier and the placental barrier respectively

Conclusions from studies of the distribution of labelled analgesics in rats using impulse counting are in general agreement with the present findings Blane and Dobbs (1967) found that the levels of etorphine which is structurally similar to diprenorphine in the brain surpassed or equalled those in blood while brain levels of dihydromorphine were only 15 % of those in blood Furthermore etorphine crossed the placenta much more easily than did dihydromorphine They also found that fecal elimination of etorphine and metabolites was important Comparatively little has been published on the distribution and elimination of fentanyl It is known however that a short time after administration of the labelled drug to rats more radioactivity is recovered from urine than from feces but that the net excreted material is approximately equally distributed between urine and feces (Wijngarden and Soudijn 1968)

The work of Adler (1963) and Herz and collaborators (see Herz and Teschemacher 1971) shows that the relative estimates of analgesic potency are different after systemic or intraventricular administration For instance while fentanyl is several hundred times more potent than morphine when given systemically it is less potent than morphine if given into the brain ventricles The present work reveals marked differences in the availability of these drugs to the CNS after systemic administration Indeed the observed differences in availability to the CNS may be taken as the explanation for the above mentioned differences in potency estimates However the exact cellular and subcellular targets in the CNS have not yet been identified and the importance of the observed differences in distribution cannot be fully evaluated at the moment It should also be noted that morphine applied into the ventricles acts more slowly than when given intravenously (von Cube *et al* 1970) showing that the intraventricular administration mode is artificial

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From the Department of Physiology Veterinärhogskolan Stockholm Sweden

Further Evidence for the Importance of CSF Na+ Concentration in Central Control of Fluid Balance

By

KERSTIN OLSSON

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Abstract

OLSSON K *Further evidence for the importance of CSF Na concentration in central control of fluid balance* Acta physiol scand 1973 *88* 183—188

Slow (0 05 ml/min) infusions of hypo- iso- and hypertonic solutions of d glucose fructose and sucrose into the CSF of the lateral cerebral ventricle of the goat were found to repress the dipsogenic antidiuretic and natriuretic responses to intracarotid infusions of hypertonic NaCl Corresponding intraventricular infusions of slightly hypotonic (0 14 M) NaCl and of 0 3 M d glucose in 0 14 M NaCl did not have this repressive effect The results are taken as support for the idea that a central receptor system which is sensitive to variations in the Na concentration of the CSF plays an important role in the control of body fluid homeostasis

The main evidence for the existence of osmoreceptors in the hypothalamic region of the brain and for a central osmometric control of the water balance has been obtained by studying the effects of alterations in the solute composition of the blood plasma (Verney 1947 Jewell and Verney 1957) In these studies the possible interaction of the blood brain and the blood liquor barriers has not been regarded However recent experiments in the goat indicate that neither changes in the blood osmolality as such nor different penetration rates across the blood brain barrier determines how effectively various blood borne stimuli cause a release of antidiuretic hormone (ADH) or elicit thirst (Eriksson Fernandez and Olsson 1971 Olsson 1972 b) This appears incompatible with the osmoreceptor theory

A possible alternative to hypothalamic osmoreceptors may be receptors near the 3rd cerebral ventricle which are influenced by the Na concentration of the cerebrospinal fluid (CSF) (Andersson 1971) Evidence has been produced that a receptor system of this kind and location is of importance not only in the control of water balance but also in the regulation of the arterial blood pressure and the renal sodium excretion (Andersson *et al* 1972) If a sodium sensitive mechanism of this kind exists a lowering of the Na concentration of the CSF ought to reduce the dipsogenic antidiuretic and natriuretic effects of a load of hypertonic NaCl which is applied outside the blood brain barrier The present results some of which earlier have been reported briefly (Olsson 1972 a) indicate that such is the case

Methods

Animals 7 adult female goats (b w 30—35 kg) were used The animals were routinely kept in metabolism cages where all experiments were conducted The experimental periods for the individual goats varied between 2 and 6 months The minimum interval between experiments in each animal was 2 days The goats were fed chopped hay every morning at 8 o clock and received 300 g of commercial grain mix (with 3 g of NaCl added) each afternoon They had free access to water at a temperature of 20±1 C

Implantation of intravascular catheters and infusion technique All animals had polyvinyl catheters permanently implanted bilaterally into the carotid artery via its superficial temporal branch as earlier described (Eriksson *et al* 1971) The catheters were used for intracarotid infusions of hypertonic NaCl at a rate of 1 5 ml/min During the infusions the free end of the vascular catheter was connected to an infusion pump via a polyethylene tubing The tubing was taped to one horn of the animal and was flexibly suspended above the goat by use of a spring balanced wheel The same technique was used for intravenous infusions Here the tubing from the infusion pump was connected to a polyethylene cannula temporarily introduced into the jugular vein

The infusions were started between 10 and 11 30 a m At this time of the day the goats had eaten to satisfaction from the morning refill of the hay bin and had also finished postprandial drinking

Implantations and infusions into the lateral cerebral ventricl To ensure free communication with the CSF over long periods of time and to make frequent intraventricular infusions possible in the non anesthetized animal a special three cannula system was developed (Akerlund An ders on and Olsson 1972) All goats had this cannula system permanently implanted into one of the lateral ventricles with the ventricular outlet placed near the foramen of Monro Th infusion technique was principally the same as that used for intravascular infusions but the rate of infusion was much slower (0 05 ml/min) In experiments involving combined intraventricular and intracarotid infusions the former were tarted 5 or 10 min ahead of the latter

Hydration A water diuresi was established by giving the goats by stomach tube 100 ml/kg b w of 38 C water 1 1/2 to 2 h before the infusions were started

Urine samples and analyses Urine was collected in 10 min samples via a retention catheter inserted into the urinary bladder Urine Na concentration was determined by use of an EEL flame photometer and an Advanced Instruments Inc osmometer was used for determina tions of blood plasma and urine osmolality The mean pre infusion plasma osmolality was 290 mosm/kg in the hydrated animal Therefore this value was used for calculations of the renal free water clearance

Results

1 *Thirst*

The influence of intraventricular infusions on the thirst response to intracarotid in fusions of hypertonic NaCl was studied with the animals in normal water balance As expected from previous experiments (Olsson 1972 b) the mere intracarotid in fusion of 1 M NaCl (1 5 ml/min) caused conspicuous cumulative drinking The 5 goats used for studies of thirst started to drink 2 to 5 min after the onset of the intra carotid infusion and consumed 1 2±0 2 liters of water during the 30 min infu ion period In contrast when 35 or 40 min intraventricular infusions (0 05 ml/min) of various saccharide solutions were started 10 min prior to a 40 min intracarotid infusion of hypertonic NaCl no water was consumed as long as the intraventricular infusion lasted

Hypo and isotonic (0 15 and 0 3 M) solutions of d gluco e and fructose were used for the intraventricular infusions in 2 of the goats Both these animals started to drink 3 to 5 min after the discontinuation of the intraventricular infusions when hypertonic NaCl still was infused into the carotid artery Hypertonic (0 5 M) sucrose olution was infused into the lateral ventricle of the remaining 3 animals None of

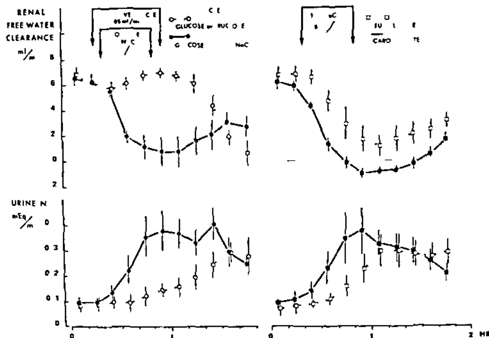


Fig 1 *Left Open symbols* Repression of the antidiuretic and natriuretic effects of intracarotid infusions of hypertonic NaCl by infusion into the lateral ventricle of iso- or hypertonic (0 3 or 0 6 M) d glucose or fructose solutions (Number of expts = 13 number of animals = 5) *Black symbols* Reappearance of these responses to intracarotid NaCl when 0.3 M d glucose is administered into the lateral ventricle in slightly hypotonic (0 14) NaCl (Number of expts = 5 number of animals = 3)

Right A comparison between the antidiuretic and natriuretic effects of intrajugular (*open symbols*) and intracarotid (*black symbols*) infusions of hypertonic (1 M) NaCl Note the more delayed and less pronounced responses to the intravenous infusions (Intrajugular Number of expts = 8 number of animals = 3 Intracarotid Number of expts = 9 number of animals = 6)

these goats consumed water during the 10 min continuation of the intracarotid NaCl infusion

All goats intermittingly consumed hay during the infusions and did not show any sign of discomfort

B ADH release and natriuresis

All 7 goats were used during hydration for studies of the antidiuretic and the natriuretic responses to intravascular infusions of hypertonic NaCl and to combined intraventricular/intracarotid infusions The intravascular infusions of 1 M NaCl (1 5 ml/min) lasted for 30 min In the experiments involving combined infusions the infusion into the lateral ventricle (0 05 ml/min) was started 5 min before and was ended 5 min after the intracarotid infusion of hypertonic NaCl

Fig 2 *Open symbols* Lack of antidiuretic and natriuretic responses to intracarotid infusions of hypertonic (1 M) NaCl performed during the simultaneous infusion of sucrose solutions (0 35 or 0 5 M) into the CSF of the lateral ventricle (Number of expts = 4 number of animals = 2) *Black symbols* Marked antidiuretic and natriuretic effects of similar intracarotid infusions performed during the infusion of slightly hypotonic (0 14 M) NaCl into the lateral ventricle (Number of expts = 5 number of animals = 4)

1 Intravascular infusions

The intracarotid infusion of hypertonic NaCl invariably had the expected strong antidiuretic effect previously shown to be due to release of ADH from the neurohypophysis (Verney 1947 Eriksson *et al* 1971) The renal free water clearance became negative within 20 min and this inhibition of the water diuresis outlasted the infusion period by 30 to 60 min The renal Na excretion rose steeply during the infusion reached its highest level about 10 min after its end and then slowly returned towards pre infusion level

The corresponding intravenous infusion of hypertonic NaCl gave weaker and more delayed antidiuretic and natriuretic responses The effects of the intracarotid and the intravenous infusions of hypertonic NaCl on renal free water clearance and sodium excretion are shown to the right in Fig 1

2 Intraventricular/intracarotid infusions

Saccharides/hypertonic NaCl When iso- or hypertonic solutions of d glucose fructose (Fig 1, left) or sucrose (Fig 2) were infused into the lateral ventricle during the simultaneous intracarotid infusion of hypertonic NaCl both the antidiuretic

and the natriuretic responses were impeded Rather the renal free water clearance rose somewhat during these combined infusions and no obvious increase in the renal Na excretion was seen until after the termination of the infusions

Control infusions Combined intraventricular/intracarotid infusions not likely to cause major reduction of the CSF Na concentration were performed as controls In 4 of the goats slightly hypotonic (0 14 M) NaCl was infused into the lateral ventricle at the usual rate (0 05 ml/min) Such infusions did not reduce the anti diuretic and natriuretic effects of hypertonic NaCl applied into the carotid artery (Fig 2) These responses also remained when intracarotid infusions of hypertonic NaCl were performed during infusions into the lateral ventricle of 0 3 M d glucose in 0 14 M NaCl (Fig 1 left) although the antidiuretic effect was somewhat weaker than that observed during the mere intracarotid infusion of hypertonic NaCl

Discussion

The current osmoreceptor theory (Verney 1947) implies that a reduced volume of osmoreceptors in the hypothalamic region of the brain is the ultimate cause of accelerated release of ADH and thirst which appear during absolute and relative dehydration However the effects of the application of various hypertonic solutions to the central nervous system of the goat both from the outside and from the in side of the blood brain barrier seem to contradict the osmoreceptor theory A rather effective blood brain barrier exists for urea glycerol fructose and Na ions (Yudile vich and de Rose 1971) Therefore their intracarotid application in equi osmolal hypertonic solutions is likely to cause the same degree of cerebral dehydration and to act as equivalent stimuli to osmoreceptors located inside the blood brain barrier Yet a rise in the carotid blood osmolality induced by urea and glycerol acts as much weaker stimulus to ADH release and thirst than the same rise elicited by NaCl or fructose (Eriksson *et al* 1971 Olsson 1972 b) The existence of osmoreceptors inside the blood brain barrier can be called in question also for another reason The infu sion of hypertonic sucrose solution into the third cerebral ventricle does not elicit thirst or ADH release in the goat in spite of the fact that the sucrose molecules do not pass into the cells (Olsson 1969)

The results presented here make it unlikely that cerebral osmoreceptors of im portance in the control of the fluid balance are located outside the blood brain barrier or in a region of the brain which lacks an effective barrier of this kind If central osmoreceptors were located outside the blood brain barrier it appears hard to explain why the antidiuretic and dipsogenic effects of intracarotid infusions of hyper tonic NaCl are repressed by slow infusions of iso or hypertonic saccharide solutions into the cerebral ventricular system However the present results seem to warrant the following conclusion

The dipsogenic antidiuretic and natriuretic effects of experimentally induced hypernatremia can be neutralized by an iso- or hyperosmotic reduction of the Na

Fig 2 *Open symbols* Lack of antidiuretic and natriuretic responses to intracarotid infusions of hypertonic (1 M) NaCl performed during the simultaneous infusion of sucrose solutions (0 35 or 0 5 M) into the CSF of the lateral ventricle (Number of expts = 4 number of animals = 2) *Black symbols* Marked antidiuretic and natriuretic effects of similar intracarotid infusions performed during the infusion of slightly hypotonic (0 14 M) NaCl into the lateral ventricle (Number of expts = 5 number of animals = 4)

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Effect of Contractions in Vitro on Glycogen Content and Glycogen Synthetase Activity in Muscle

By

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Abstract

ADOLFSSON S *Effect of contractions in vitro on glycogen content and glycogen synthetase activity in muscle* Acta physiol scand 1973 *88* 189—197

The influence of contraction on the content of glycogen in muscle as well as on the activity of the glycogen synthetase enzyme (EC 2 4 1 11)—rate limiting for glycogen synthesis—has previously been investigated *in vivo* In the present study this influence was studied *in vitro* where interference of epinephrine and insulin on the the effects of contraction is more easily excluded Electrically stimulated intact levator ani muscle preparations from prepubertal rats were used (Arvill Acta endocr 1967 *56* suppl 122) Glycogen content decreased during 15 min of intermittent tetanization (10 s/min) There was no change in synthetase activity during the contractions For the period following contractions an increase in the per cent active I form of the enzyme was found after 1 ? 10 and 180 min Resynthesis of glycogen was shown after 30—180 min An inverse relation between % I form and glycogen content was found when data from 1—10 min after contractions were plotted together with the data from unstimulated muscles During repeated single twitches (1/s) for 60 min there was an enhanced penetration of xylose ^{14}C and a higher rate of incorporation of glucose ^{14}C into glycogen compared to control muscles The present results are in good agreement with those previously obtained *in vivo* Thus the influence of contractions *in vivo* could be demonstrated also *in vitro*

The glycogen synthetase enzyme (UDP glucose α 1 4 glucan α glucosyltransferase EC 2 4 1 11) is considered rate limiting for glycogen synthesis (Villar Palasi and Larner 1961) In muscle tissue the influence of physiological stimuli like insulin and epinephrine on glycogen synthesis and synthetase activity has been studied *in vivo* (Danforth 1965 Søvik 1966 Goldberg *et al* 1967 Adolfsson 1972 a) and *in vitro* (Villar Palasi and Larner 1961 Craig and Larner 1964 Danforth 1965 Adolfsson 1972 b) The influence of muscular contractions on regulation of glycogen synthesis in muscle however has only been investigated *in vivo* where it can be difficult to exclude a *regulatory influence by e g the hormones mentioned above The in vivo* studies have been performed on leg muscle of anesthetized mouse and rat after electrical stimulation (Danforth 1965 Stanelom and Piras 1969 Piras and Staneloni 1969) as well as on leg muscle of man after bicycle exercise (Hultman *et al* 1971) In these studies muscular contractions were found to induce the following pattern

of effects 1 during contraction glycogen content was decreased and there was no activation of the synthetase enzyme 2 following contraction an increase in synthetase activity by means of a conversion of the D form of the enzyme (dependent on glucose 6 phosphate) to active I form (independent of glucose 6 phosphate) was found after a lag phase of 1—3 min and 3 during a period following contractions there was a pronounced resynthesis of glycogen In addition it is well known from other studies that the uptake of glucose in muscle is stimulated during and after contractions (for ref see Arvill 1967) The mechanisms behind this pattern of effects are not fully elucidated The present study was undertaken to investigate if a similar pattern could be demonstrated under *in vitro* conditions

Electrically stimulated intact levator ani (LA) muscle preparations from prepubertal rats were used (Arvill 1967) The two forms of the synthetase enzyme, I form and D form were measured as well as the glycogen content and the rate of incorporation of glucose ^{14}C into glycogen and the accumulation of xylose ^{14}C in the intracellular water

Some preliminary data have been presented in a previous communication (Adolfsson 1970)

Methods

Intact LA muscle preparations (Arvill and Ahren 1966) from prepubertal male Sprague Dawley rats weighing 50—60 g were used The rats were killed by cervical fracture Incubations were performed at 37° C in Krebs Ringer bicarbonate buffer pH 7 4 containing glucose 2 mg/ml The buffer was gassed with a O_2—CO_2 mixture (95 5 v/v) equilibrated with water In all experiments 25 ml vessels with 4 ml buffer were used for incubations except when incubations were performed for 30 and 180 min after contractions In these experiments 10 ml vessels with 1ml buffer were used in accordance with the previous routine (Arvill and Ahren 1966 Adolfsson 1972 b) In some experiments xylose ^{14}C or glucose ^{14}C were added to the incubation medium details are given in the legends to Table II and III

The LA muscle preparation is ring shaped and a part of the ring consists of the bulbocavernosus muscles In both control and electrically stimulated muscles this part of the ring was hung up on one of the electrodes in the incubation vessel the other electrode was in the incubation medium (Arvill 1967) The muscles were electrically stimulated by impulses of 9 V and 1 ms duration Usually intermittent stimulation of 40 Hz during 10 s per min was given for various periods In some experiments (Table III) continuous stimulation of 1 Hz was used

At the end of incubation the muscle preparation was rapidly blotted on filter paper and frozen in Frigen ($CFCl_3$) chilled with solid CO_2 The LA muscle was cut off from the bulbocavernosus muscles and weighed In some experiments the LA muscles were divided into 2 parts one for determination of glycogen the other for determination of synthetase activity When the muscle preparations were used for determination of accumulation of xylose ^{14}C they were not frozen in Frigen but rapidly washed in ice-chilled 0 9 % NaCl and blotted on filter paper before being weighed and homogenized in 10 % trichloro acetic acid

Glycogen synthetase activity was essentially determined according to Villar Palasi *et al* (1966) with the modifications previously described (Adolfsson 1972 b) The supernatant after centrifugation (5000 × g) of a homogenate of LA muscle in Tris buffer (pH 7 8 50 mM Tris 5 mM EDTA 10 mM NaF) was used The final dilution of the muscle tissue in the assay (pH 7 8) was 1 100 (w/v) Enzyme activity was measured in absence and presence of 10 mM glucose 6-phosphate and referred to as I form and (I + D) form activity respectively Values are given in mU/g wet tissue (1 mU = 1 nanomole of glucose incorporated into glycogen from UDP glucose per min) The activity of the I form is also expressed in per cent of (I + D) form activity which was unchanged during all experimental conditions

Glycogen was isolated from KOH digest of muscle by ethanol precipitations and determined with an all-enzymatic method (for details see Adolfsson 1972 a) When only part of the LA muscle (5 mg) was available for glycogen determination the volume of the reaction mixture

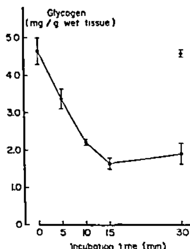


Fig 1

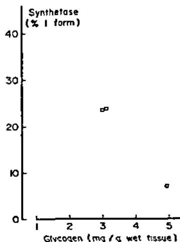


Fig 2

Fig 1 Influence of various periods of contractions *in vitro* on the glycogen content of rat LA muscle Muscles were hung up on electrodes and incubated for the periods indicated For each min of incubation electrical stimulation of 40 Hz was given during 10 s Each point represents the mean value of 4 muscles S E × 2 is indicated by the length of the vertical bars The point at 0 min shows the glycogen content at start of incubation The solitary point indicates the glycogen content of muscles that were hung up on electrodes and incubated for 30 min without electrical stimulation

Fig 2 Relation between glycogen content and the percentage of synthetase in the I form in LA muscles from fasted and fed rats stimulated electrically for 5 min as described in the legend to Fig 1 Squares represent control muscles open triangles show stimulated muscles 1 and 2 min after contraction and filled triangles indicate stimulated muscles 10 min after contraction Fasted rats were deprived of food for 18—20 h For further information see Table I

was reduced from 1600 µl to 500 µl Rabbit liver glycogen (Sigma, type III) was used as standard Glycogen content is expressed in mg/g wet tissue The radioactivity of the glycogen from incorporated glucose-^{14}C was determined by liquid scintillation technique and expressed as CPM/mg wet tissue (*cf* Adolfsson *et al* 1972) Accumulation of xylose-^{14}C into the intracellular fluid was determined and expressed as described by Arvill (1967)

All chemicals were of analytical grade UDP glucose ^{14}C (U) (used in the synthetase assay) was obtained from NEN Chemicals (Boston Mass USA) Glucose ^{14}C (U) and D xylose ^{14}C (U) were from the Radiochemical Centre (Amersham England)

Student's t test was used to compare differences between mean values For the data of Table I and II the one way analysis of variance followed by Student—Newman—Keuls test was used (*cf* Woolf 1968) A p value of 0.05 is considered significant in this study

Results

The influence of electrical stimulation on glycogen content of LA muscles from fed rats is illustrated in Fig 1 A tetanic contraction of 10 s was induced during each min of incubation A significant decrease in glycogen content was observed after 5 min. After 15 min of incubation glycogen was reduced to 1.7 mg/g wet tissue from a resting value of 4.6 mg/g no further reduction was observed after 30 min To investigate if it is possible to get lower levels of glycogen in this muscle the same kind of stimulation was given to LA muscles incubated for 15 min in an anaerobic

TABLE I Glycogen synthetase activity and glycogen content in rat LA muscle 0–10 min after contractions in vitro

	Fed rats			Fasted rats			
	Control	Time after contraction (min)		Control	Time after contraction (min)		
		0	2		0	1	10
Synthetase							
% I form	7.4±0.3	9.8±0.6	19.6±1.4	21.7±1.6	19.0±0.5	30.2±3.3	30.1±2.9
I form mU/g	65±1	94±6	170±13	172±14	200±9	254±33	272±46
(I+D) form mU/g	887±37	1031±91	915±53	864±83	1015±46	914±107	895±91
Glycogen mg/g	4.69±0.18	3.09±0.37	3.11±0.28	3.47±0.33	2.09±0.26	1.97±0.03	2.09±0.29

LA muscles were stimulated electrically for 5 min as described in the legend to Fig 1 Synthetase activity and glycogen content were measured directly after stimulation or 1, 2 and 10 min after Control muscles were incubated for 5 min without stimulation There are 3–4 observations in each group Mean values ± S E are given Fasted rats were deprived of food for 18–20 h

medium saturated with N_2 instead of O_2 A glycogen level of 0.90±0.05 (4 muscles) was obtained With continuous tetanization a low glycogen content was also obtained under aerobic conditions After 15 min of tetanic contractions glycogen was reduced to 0.72±0.10 mg/g Such a continuous tetanization was not employed however, when it was found that the recovery after stimulation in terms of resynthesis of glycogen was less pronounced than after the intermittent stimulation described above Single twitches induced by continuous stimulation with 1 Hz decreased the glycogen content quite slowly as shown in Table III

Glycogen synthetase activity and glycogen were measured on LA muscles intermittently tetanized (10 s/min) for 5 min Muscles from fasted and fed rats with a glycogen content of 3.5 and 4.7 mg/g wet tissue respectively were used The results are shown in Table I A lower resting level of % I form in the synthetase enzyme was observed in fed rats than in fasted rats Immediately after tetanization there was no change in % I form compared to controls When determined 1, 2 and 10 min after contraction the % I form was markedly increased The relation between % I form and glycogen content in individual muscles is plotted in Fig 2 Values obtained immediately after contraction are not included in this figure The symbols appear to be located on the same line indicating an inverse relationship between % I form and glycogen content

The resynthesis of glycogen after contraction was also studied The results of intermittent stimulation (10 s/min) for 15 min and subsequent incubation for 30 and 180 min are shown in Table II Compared to the glycogen content immediately after contractions there was a small but significant increase after 30 min The increase in glycogen content was associated with an increased incorporation of glucose-^{14}C into glycogen When measured 180 min after contraction the glycogen content of the stimulated muscles was found to be further increased although it was still

TABLE II Glycogen synthetase activity and glycogen content in rat LA muscle 1–180 min after contractions *in vitro*

		Time after contractions (min)					
		1 min		30 min		180 min	
		Control	Contraction	Control	Contraction	Control	Contraction
Synthetase	I form	14.1±1.7	36.5±0.7	–	–	8.4±1.2	16.1±1.7
Glycogen mg/g wet tissue		4.84±0.38	1.70±0.11	4.29±0.6	2.31±0.19	4.14±0.23	3.61±0.24
Incorp of glucose C CPM/mg wet tissue		–	–	49±18	810±107	–	–

LA muscles from fed rats were stimulated electrically for 15 min as described in the legend to Fig 1 After stimulation the muscles were transferred to new vessels without electrodes and incubated for 30 and 180 min in presence of glucose 2 mg/ml For 30 min incubations D glucose C (U 2–4 mCi/mmole) was added to the incubation medium (2 μCi ml) and the incorporation into glycogen determined During the conditions studied there was no change in synthetase (I+D) form activity There are 4 muscles in each group Mean values±S E are given

TABLE III Intracellular accumulation of xylose ^{14}C and incorporation of glucose C into glycogen in rat LA muscle during contractions *in vitro*

	Control	Contraction
Accumulation of xylose C $\frac{100 \times \text{CPM/ml intracellular water}}{\text{CPM/ml medium}}$	11.5±1.0	50.5±3.9
Incorporation of glucose C CPM/mg wet tissue	6?±5	134±11
Glycogen mg/g wet tissue	3.10±0.26	1.90±0.10

LA muscles from rats deprived of food for 18–20 h were stimulated electrically with 1 Hz for 60 min Xylose C or glucose C was present in the incubation medium during the contractions D xylose C (U) was used at a concentration of 1.3 mM and an activity of 0.5 μCi ml Corresponding values for glucose C are given in the legend to Table II Intracellular accumulation of xylose C is expressed as the radioactivity in 1 ml of the intracellular fluid in per cent of the radioactivity in 1 ml of the medium There are 4–5 muscles in each group Mean values ±S E are given

below that of unstimulated muscles incubated for the same time Synthetase activity was also determined after 180 min and a higher % I form was present in contracted muscles than in controls Considering the difference in glycogen content between the 2 groups of muscles this higher % I form is compatible with the inverse relationship between % I form and glycogen shown in Fig 2

The permeability of the muscle cell membrane to sugars was studied by measuring the accumulation of xylose ^{14}C in the intracellular water during 60 min of incubation (Table III) Electrical stimulation of 1 Hz was given As mentioned previously the decrease in glycogen was slow with this moderate rate of stimulation The accumulation of xylose ^{14}C was increased during the period of contraction (Table III) The

incorporation of glucose ^{14}C into glycogen was also increased during similar contractions but the increase was small compared to that observed after tetanic contractions (Table II)

Discussion

In the present study electrical stimulation *in vitro* was found to increase the percentage of active I form in the synthetase enzyme when measured 1 min or longer after stimulation During stimulation there was no increase in I form Glycogen content was decreased by electrical stimulation and a resynthesis was found after stimulation In accordance with previous *in vivo* studies (Danforth and Harvey 1964) an inverse relation between the % I form and the glycogen content could be demonstrated When % I form was plotted versus glycogen content data from unstimulated muscles and muscles incubated for 1 2 and 10 min after electrical stimulation appeared to fit the same line There was no indication of a change in the relation between % I form and glycogen content This is in contrast to the change observed after stimulation by insulin or epinephrine (*cf* Danforth 1965)

An explanation for the influence of the glycogen level on the synthetase enzyme is offered by the findings of Villar Palasi and Larner (1966) who reported an inhibition by glycogen of the activity of the phosphatase enzyme catalyzing the conversion of synthetase D form to I form When glycogen is reduced by contractions the inhibition of the phosphatase should decrease and give rise to a higher level of I form However as shown in the present study the expected increase in I form cannot be demonstrated immediately after tetanic contractions but after a lag phase of 1 min Similar results have also been obtained *in vivo* (Danforth 1965 Stanelon and Piras 1969)

The reason for the lack of increase in I form during and immediately after contractions could be that the release of the inhibition of the phosphatase by glycogen is counteracted in some way and/or that mechanisms acting to decrease the I form are engaged under these conditions The latter possibility is supported by the findings of Stanelon and Piras (1969) that tetanic contractions very rapidly (within 5 s) decrease the I form This was demonstrated *in vivo* in muscles with high % I form induced by previous contractions No such decrease in I form by contraction was seen in the present study which might be due to the fact that the level of I form before contraction was only half of that seen in the experiments cited However at similar levels of glycogen the ratio of % I form to glycogen was much lower during tetanic contractions than 2 and 10 min after contractions (Table I 9 8 % I form to 3 1 mg/g glycogen *vs* 19 6 to 3 1 and 19 0 to 2 1 *vs* 30 1 to 2 1 for fed and fasted rats respectively) Such a decrease in the ratio between % I form and glycogen is also observed during stimulation by epinephrine (Danforth 1965) It has been shown that the effect of epinephrine is mediated by an activation of the protein kinase catalyzing the conversion of synthetase I form to D form The protein kinase is activated by an increased level of cyclic AMP as well as by transformation to a

form less dependent on cyclic AMP for full activity (for ref see Shen *et al* 1970) Similar mechanisms for the decrease in synthetase I form are probably not in function during muscular contractions as there is no influence on the level of cyclic AMP (Posner *et al* 1965) and no change in the cyclic AMP dependency of the protein kinase during contractions (Stanelom and Piras 1969) Concerning the possibility that the lack of increase in I form during contractions is due to inhibition of the phosphatase enzyme catalyzing the conversion of synthetase D form to I form the results from recent studies on the regulation of glycogen metabolism in the liver may be considered It has been shown that the phosphatase enzyme is inhibited by the a form of the phosphorylase enzyme (Stalmans *et al* 1971) As phosphorylase a form is markedly increased in muscle during contractions it would be very interesting to determine whether this mechanism is operating also in muscle tissue to decrease synthetase I form

Muscular contractions *in vitro* were not found to influence the total enzyme activity (I+D) form Similar results have been obtained *in vivo* *e g* in man after a single bout of exercise (see Hultman *et al* 1971) and in rat after tetanization (Stanelom and Piras 1969) This is in contrast to the increase in total synthetase activity with training observed in guinea pigs exercised regularly for 3 weeks on a tread mill (Jeffress *et al* 1968) and in man undergoing a training program (varsity wrestling) for 5 months (Taylor *et al* 1972)

In the present study an increase in the permeability to xylose ^{14}C was found during moderate contractions of 1/s This result is in good agreement with those of a similar study by Arvill (1967), who in addition showed increased disappearance of glucose from the incubation medium when LA muscle preparations were stimulated electrically In other *in vitro* studies an increased intracellular accumulation of 3 methylglucose has been demonstrated during repeated contractions (Holloszy and Narahara 1965) These data indicate that at least under single twich contractions there is an increased transport of glucose across the cell membrane The increase in uptake of glucose and the increased degradation of glycogen give rise to a higher level of glucose 6 phosphate in the muscle during contractions As discussed by Piras and Stanelom (1969) the level of glucose 6 phosphate could be high enough to activate the D form of the synthetase enzyme and thus stimulate glycogen synthesis in the absence of an increase in the I form As shown in the present study there is an increase in incorporation of glucose ^{14}C into glycogen during the contractions A similar increase has been reported for the isolated perfused rat diaphragm muscle when this preparation was electrically stimulated to contraction 12 times/min (Beloff Chain *et al* 1971)

When the rate of incorporation of glucose ^{14}C into glycogen was determined in muscles after the period of contraction an increase above control levels was found This increase was higher than that observed during the contractions From previous studies it is known that membrane transport of glucose is stimulated for at least 3 h after muscular contractions *in vitro* (Arvill 1967) During the 3 h studied in the present investigation the increase in glycogen synthesis after contraction was not

large enough to restore glycogen to the level observed before contraction Similar results have been reported for the *in situ* preparation of the gastrocnemius plantaris muscle group of the dog stimulated 5 times/s for 60 min (Chapler and Stainsby *1968*) *In that* preparation complete recovery of the glycogen was not obtained with in the 2 h studied For comparison it could be mentioned that it takes 24 h to restore the glycogen content in leg muscle of man after a strenuous bicycle exercise (Hultman *et al* 1971) However the glycogen level is normally 4—5 times higher in man than in rat In these *in vivo* studies it is difficult to exclude a stimulation by insulin during the recovery period *In* an *in vitro* system influence of insulin is very unlikely to account for the resynthesis of glycogen The present *in vitro* results indicate that stimulation of glycogen synthesis and activation of the synthetase enzyme are induced by muscular contractions independent of other factors affecting glycogen metabolism The isolated LA muscle preparation seems to be well suited for further characteriza tion of the mechanisms behind the effects of contractions

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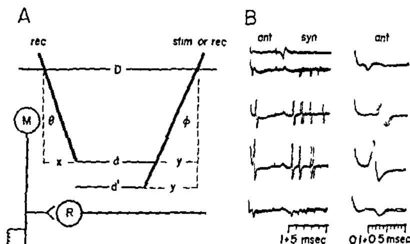


Fig 1 Schematic representation of experimental arrangement The recording (rec) and the stimulating (stim) microelectrodes were introduced at different angles (ϴ and ϕ respectively) The distance (d) between their tip was calculated from their distance (D) at the surface with correction for the rostro caudal component of the electrode tracks at their respective depths e g x and y or x and y The motoneurone (M) axons which evoked synaptic responses in the Renshaw cells (R) via their axon collaterals were activated antidromically by stimulation of peripheral nerves and/or ventral roots In B the cord surface response (top record) and the extracellular spikes of a Renshaw cell evoked antidromically (ant) from different sites in the spinal cord and synaptically (syn) by stimulation of PBSt are shown for slightly different depths of the recording electrode The parallel changes in amplitudes of the antidromic and the synaptic responses show that they were recorded from the same cell The antidromic spikes are shown at a faster sweep speed in right hand records Time calibrations are indicated by shorter (0 1 or 1 ms) and longer (0 5 or 5 ms) bars

paralyzed with gallamine triethiodide (Flaxedil) and artificially ventilated Temperature blood pressure and end tidal CO_2 were monitored and regulated as described previously (*cf* Hultborn *et al* 1971 a Jankowska and Roberts 1972 a)

Experimental procedure The techniques used were identical in principle with those described by Jankowska and Roberts (1972 a *cf* their Fig 1) for antidromic activation of Ia inhibitory interneurones The activity of individual Renshaw cells was recorded extracellularly with one of two microelectrodes inserted into the spinal cord The second microelectrode was used for either recording or stimulation and could be moved independently The 2 electrodes were introduced at different angles relative to the axis of the cord usually at −20 and +20 The distances between the tips of the two electrodes in the rostro-caudal direction were calculated from their distances at the surface D in Fig 1 A from which were subtracted the rostro-caudal components of the trajectories of the electrodes as they penetrated the cord (x and y) The electrodes had tip diameters of 1 5—2 5 μm They were filled with 3 M NaCl solution and had resistances of 2—6 MΩ The electrode used for stimulation was shielded as described by Eide (1971) Short rectangular pulses (0 1 ms) were used for stimulation The intensity of the current was recorded (see Fig 1 of Jankowska and Roberts 1972 a) as the voltage drop across a 100 Ω resistance placed in the return path to ground

Renshaw cells were sought in S1 (preferably) and in L7 segments in order to make it possible to activate them by stimulation of VRs (that is by all axon collaterals supplying them) as well as by stimulation of different nerves to define their input Another reason for selecting cells in S1 was to have the maximal length of the lumbar enlargement to investigate their rostral projection

The stimulating electrode was introduced into either the same segment or one or two situated more rostrally The location of its tip within the spinal cord were determined in relation to the locations at which antidromic motor fields were recorded (*cf* Fig 6) and from the subsequent histological control After a reconstruction of the electrode tracks the stimulation sites were determined from the known distances from the surface and the coefficient of shrinkage of the preparation (about 15) during the histological procedures

The following records were always taken in parallel a) responses to microelectrode stimulation (Fig 1 B ant) b) responses to stimulation of motor axons which evoked a synaptic response of the Renshaw cell (Fig 1 B syn) c) potentials from the lateral surface of the spinal cord (indicating arrival of the volley in the motor axons uppermost record in Fig 1 B) and d) the amplitude of current pulses passed through the stimulating electrode (Fig 3 B and E) A parallel microelectrode and synaptic activation of a selected Renshaw cell was used to insure that the recording electrode was well positioned in relation to it that the cell was not damaged and that all the records were taken from the same cell The latter was indicated by a similar shape of the spike potentials evoked synaptically and antidromically and by parallel changes in their amplitudes as illustrated in Fig 1 B

Results

1 *A comparison between antidromic and synaptic activation of Renshaw cells*

To enable differentiation between antidromic and synaptic activation of Renshaw cells by microelectrode stimulation the first series of experiments was designed as follows Cells located in S1 and activated by volleys in S1 VR motor fibres but *not by those in L7 V R* were selected for the analysis The electrical pulses were applied through the stimulating microelectrode at least half a segment from the rostral border of S1 in order to avoid stimulation of axon collaterals of motor fibres originating in S1 and projecting rostrally For the range of stimuli used there was never any indication of indirect excitation of S1 motoneurones via some fibres in more rostral segments and as a consequence of activation of Renshaw cells by collaterals of these motoneurones

Under these conditions the microelectrode stimulation in L7 always evoked only a single spike while the motor fibre stimulation induced a typical series of spikes with decreasing frequency (Renshaw 1946) A reduction of the number of synaptically evoked spikes to 2—3 and occasionally to one was possible when threshold stimuli were used but it was always combined with a pronounced increase in their latencies and an irregularity in their appearance On the other hand the single spike responses evoked by microelectrode stimulation in L7 occurred with great regularity and little ($<$ 0 05 ms) variability of latencies Therefore they could hardly be considered as the result of a liminal synaptic activation by the above mentioned hypothetical indirect excitation of S1 motoneurones

Their antidromic character was always confirmed by the collision test whenever it was applied This required finding the shortest time intervals at which the spikes evoked by microelectrode stimulation could follow other spikes generated in the soma region If the applied pulses evoked a *synaptic excitation* this time interval would correspond to the refractory period of the soma or of the initial segment of the axon This was determined by delivering a maximal stimulation to the motor axons after the microelectrode stimulation as shown in Fig 2 A and B In A each dot represents a spike antidromic (○) or synaptic (●) and the shortest time interval between them (hatched) is illustrated by the bottom record in B The mean value of refractory periods for all cells tested was 0 87 $\pm$ 0 14 (S D) ms This was only 0 13 ms shorter than the mean interval between the first two spikes in the initial high frequency burst (*cf* middle record in B) which was 1 00 $\pm$ 0 16 S D ms The

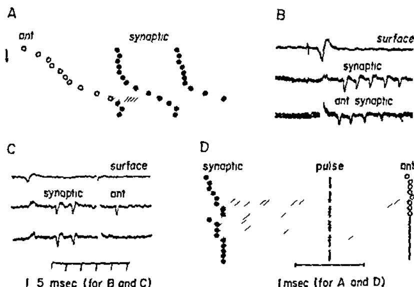


Fig 2 Collision test A series of time intervals at which spikes evoked synaptically (●) by stimulation of ABSm (the arrow indicates the onset of the cord surface potential) followed an antidromic spike (O) Each row of data represents one record The hatched area indicates the shortest interval equivalent to the refractory period of the soma The corresponding records are in B where the top record is the cord surface response the middle record is a synaptic response alone and the bottom record is the synaptic response preceded by the antidromic spike In D there is a plot similar to A showing at which time intervals stimulus pulses (dashed line) can evoke a response (O) following the synaptic spikes (●) The data were chosen to show the shortest interval (hatched) at which the antidromic response did appear and the longest interval between the synaptic and the expected time of occurrence (solid line) of an antidromic spike at which it was missing

time interval between the *antidromic invasion* of a cell and a preceding spike generated at the soma should be equal to twice the antidromic conduction time over a given length of the axon plus the refractory period of the axon near the stimulating electrode For slow conduction velocities and/or sufficiently long distances between the stimulation sites and the cell body this time period should clearly exceed the refractory period of the soma since the latter is of the same order as the refractory period of the axon (*cf* Jankowska and Roberts 1972 a and below) Because of very short intervals between the first few successive spikes in the synaptic responses of Renshaw cells the antidromic invasion had to be tested with respect to one of the later spikes in a train unless only one or two appeared at threshold stimulation of the motor axons In both cases the conditioning synaptic spikes appeared at irregular times after the applied shocks and a number of single sweep records had to be taken to find the shortest interval between these spikes and the appearance of antidromic spikes The results of such testing are shown in Fig 2 D with sample records in C The records illustrate the shortest interval at which the second spike occurred (2 75 ms middle trace) and the longest taking into account when it should be evoked after the stimulus pulse at which it was missing (2 90 ms bottom trace)

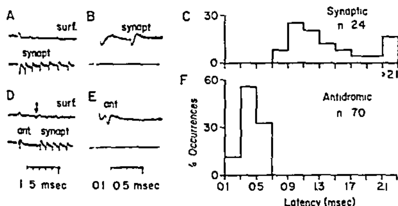


Fig 3 Comparison of latencies of responses to synaptic and to antidromic activation by microelectrode stimulation at sites within two mm from the soma of Renshaw cells Record in A show responses of Renshaw cell (lower trace) evoked synaptically by microelectrode stimulation Records in D show responses evoked antidromically (ant) and synaptically following stimulation of the peripheral nerve (arrow) The first spikes in A and D are shown at a faster sweep speed in B and E along with the amplitudes of the stimulus pulses (lower records retouched) The distributions of latencies of synaptic and antidromic responses for all data are given in C and F

Assuming that microelectrode stimulation generated spike potentials in the axons of Renshaw cells with the same latent period as that found for Ia inhibitory interneurones (Jankowska and Roberts 1972 a) i e with about 0 2 ms the antidromic conduction time could be estimated from the latency of the antidromic responses Subtraction of twice this time from the shortest interval between the spikes evoked synaptically and antidromically gives in turn approximate estimates of the refractory periods of Renshaw cell axons These were calculated for 11 cell and ranged between 0 75 and 1 25 ms

Besides their different discharge characteristics (single versus a series of spikes) the antidromic and the synaptic responses differed also in their latencies The spikes evoked antidromically appeared at least 0 1 ms earlier than any synaptic responses induced by stimulus pulses applied at the same distance from the cells This is illustrated in Fig 3 which shows the synaptic (A B) and the antidromic (D E) responses evoked by pulses delivered at different depths along the same electrode track The histograms (C F) give the distributions of latencies for synaptic and antidromic responses obtained from distances less than 2 mm The shortest latencies for synaptic responses were 0 78 ms These were always longer even at a maximal stimulus strength than the minimum central delay of the same Renshaw cell responses to peripheral nerve volleys the difference being attributable to the latent period for excitation of the fibres by electrical pulses

Thus it appeared that *the single spike response with a regular short (< 0 7 ms for 2 mm distance) latency clearly differentiates the antidromic from the synaptic response of Renshaw cells* In view of this and the negligible risk of mistaking one for another most of the experiments were performed under less rigorous conditions The records were taken from Renshaw cells located in L7 as well as in S1 and the

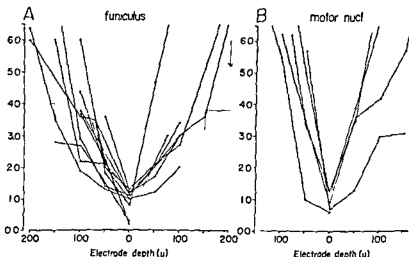


Fig 4 Threshold for antidromic activation as a function of the distance from the fibre In A and B the minimum stimulus strengths in μA (ordinates) required for 0 1 ms pulses to activate axons of Renshaw cells are plotted for different positions of the stimulating electrodes above (+) or below (−) the position where the threshold was lowest Only cells with minimum threshold ⩽ 1 3 μA were selected Data for stimulus sites in the white matter are in A and for those in the motor nuclei are in B Data points obtained from a given electrode track are connected by solid lines Since the stimulation was delivered at fixed depth increments the actual minimum threshold point was not tested in every case a dashed line was used to connect two points that clearly seemed to straddle the actual minimum The arrows in A denote the maximum distances from the minimum threshold points at which the majority of the fibres tested could be activated with currents of either 6 or 4 μA

attempts to activate them were made from both the same and the more rostral segments

2 *Interactions between the antidromic and the synaptic responses*

It has been shown that repetitive discharge in the lobster stretch receptor neurone can result from depolarization of the firing zone by delayed invasion of dendrites and their responses asynchronous with the soma responses (Grampp 1966a b) This has not been found to be the case in the Renshaw cells in which the antidromic impulses evoked only single spikes in the soma while under the same conditions orthodromic volleys generated a train of spikes However single antidromic response did not exclude that dendritic invasion might add to the prolonged transmitter actions to evoke repetitive discharge of Renshaw cells If this were the case the antidromic activation of these cells should facilitate weak synaptic responses and a decrease in the latencies of the immediately following synaptic spikes and/or an increase in their number might occur Neither of these phenomena was observed On the contrary the antidromic response nearly always lengthened the interval between the first two or three subsequently elicited orthodromic Renshaw responses and/or reduced the firing index of such responses This was seen in cases in which the orthodromic response was evoked by both barely and suprathreshold stimulation of the peripheral nerves

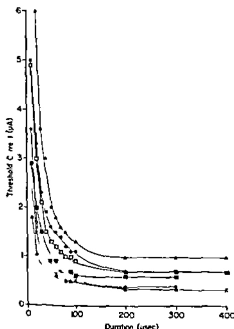


Fig 5 Strength duration curves Data for seven fibres stimulated in either gray or white matter with thresholds less than 1 μA

3 *Axonal projections*

By tracking systematically within the spinal cord it was usually possible to find a number of sites from which the selected Renshaw cells were activated antidromically and in this way to define their projection The distance between the tip of the stimulating electrode and an axonal branch was estimated from changes in threshold current with changes in the position of the electrode The lowest thresholds were less than 0 5 μA similar to the values reported for Ia inhibitory interneurones (Jankowska and Roberts 1972 a) Thus for Renshaw cells it was also assumed that if the electrode were nearly touching the fibre close to one of the nodes of Ranvier the threshold for generation of a spike potential would be about 0 1 μA With this assumption and the data in Fig 4 A and B which relate threshold current to the distance of the electrode tip from the depth at which the threshold was minimum it can be concluded that a threshold current of 4 μA would correspond to a maximal distance of about 150 μm from the node and that of 6 μA to about 200 μm (arrows) If the electrode tracks passed the fibres between the nodes the shortest distances between these tracks and the fibre would be less than these estimates The relationship between threshold strength and duration of the pulses (Fig 5) was measured for the axons of seven Renshaw cells and was found to be approximately the same as for Ia interneurones

Thirty five Renshaw cells were investigated of which 31 could be activated anti

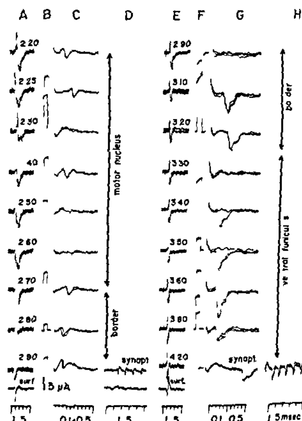


Fig. 6. Responses of 2 Renshaw cells (A—D and E—H) evoked by current pulses applied in various regions of the spinal cord. The depths of stimulation are indicated in A and E above field potentials recorded at the same electrode positions at which current pulses were applied and from which the responses to the right were recorded. B and F: amplitudes of threshold current pulses (retouched; cf. calibration in B). C and G: extracellular responses of Renshaw cells with the end of the shock artifact to the left; all are antidromic except that at depth 4.2 mm in G. D: typical synaptic response of the same cell to peripheral nerve stimulation and the corresponding record from the lateral surface of the spinal cord. H: the same synaptic response as in G but with slower sweep speed. The positions of the stimulating electrodes (0.1 and 1.7 mm rostrally) at a depth of 3.0 mm from the somas of the two cells respectively) were in motor nuclei, ventral funiculus and border region between them as indicated in D and H. Changes in amplitudes of the recorded spike potentials were due to some movements of the recording electrode in relation to the cells while the stimulating electrode penetrated the cord. In all these cases the antidromic responses changed in parallel with synaptic responses of the cells (as in Fig. 1 B).

dromically. Most of them were located in S1 and were excited following stimulation of PBSt and/or ABSm nerves. The remaining cells were excited also from G S, PL and FDL or else had their input only from the latter group of nerves.

Funicular projections. The regions from which Renshaw cells were activated antidromically were defined by subsequent recording of field potentials evoked by stimulation of motor nerves and by histological verification of the relevant electrode tracks. When the stimulus strength was below 6 µA and the electrode tips were clearly within or ventro-medial to motor nuclei, it was concluded that the stimulated axonal branches projected to the motor nuclei or were running in the ventral funiculus respectively. In intermediate cases, when the lowest threshold for antidromic activation of the cells was found at electrode tip positions less than about 200 µm from the ventro-medial border of the motor nuclei, these regions were classified as "border" regions.

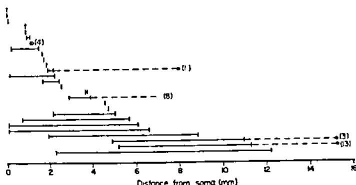


Fig 7 Distances from the soma at which 27 Renshaw cell axons were activated antidromically from within the white matter Vertical bars alone or connected by solid lines indicate the shortest and the longest distances Circles represent the longest distances at which activation was attempted but not successful the number of tracks is given in parentheses Filled circles indicate that the tracking covered the area in which Renshaw axons should be located sufficiently to exclude projection to the given level open circles indicate that the tracking was not conclusive

Fig 6 shows records of spike potentials evoked antidromically in two cell by current pulses applied in 2 different electrode tracks passing through the motor nuclei (columns A—D) and medially to them (columns E—H) Antidromic responses of the first cell were evoked from dorsal (depths 2 2 mm) and ventral (depths 2 4—2 6 mm) parts of the motor nuclei and from a border region just medio ventral to them The various latencies of the antidromic responses indicate that different axonal branches were stimulated at different depths only 50—100 μm away The second cell was activated by rather strong current pulses applied in the border region (depths 3 1—3 2 mm) and with a lower threshold from the ventral funiculus (depth 3 5 mm) Two different axonal branches were probably stimulated at these electrode positions as indicated by the absence of a response at depth 3 3 mm and longer latencies at 3 1—3 2 mm At the deepest electrode position (depth 4 2 mm) a synaptic response was evoked

Funicular projections were found in all but 2 of the cells activated antidromically On the basis of histological reconstruction of those electrode tracks in which the axons were excited within the white matter the approximate locations of 9 fibres were defined (Fig 8 F) All of them were running in the ventral funiculus near the zone through which ventral root fibres were passing

Estimation of the distance over which Renshaw cell axons project Previous estimates of the maximal distances between Renshaw cells and their target cells were based on measurements of the distances between the level of emergence of the efferents exciting a given population of Renshaw cells and the location of cells inhibited by them (Ryall *et al* 1971 Hultborn *et al* 1971 b d) for the motor axon collaterals should terminate on Renshaw cells within quite narrow limits (Ryall 1970 *cf* also

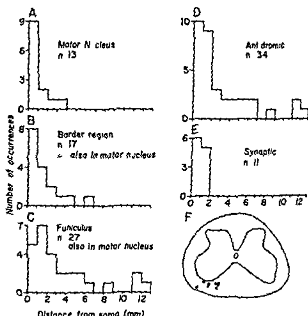


Fig 8 A comparison of the lorgest distances from which single Renshaw cells were activated antidromically from sites within the motor nuclei (A) the ventral funiculus (C) and the border region between them (B) Hatched areas indicate that cells activated from a given distance in the funiculus or the border area were also activated from *some sites within the motor nuclei* The longest distances from which single cells were activated antidromically or synaptically regardless of stimulation region are shown in D and E The localization of the electrode tips from which 9 Renshaw *cell* axons running in the white matter were activated are shown in F All are projected to a cross section of the spinal cord in rostral L 7 through which most of the tracks passed

below) From these measurements it appeared that Renshaw cell axons project over a distance of at least one segment (Hultborn *et al* 1971 b d) or 5 5 mm (Ryall *et al* 1971) However, in view of a relatively long intraspinal trajectory of motor axons the distances based on reference points such as the level of their emergence or the border between two dorsal roots are only approximate From the measurements of the distance from which single Renshaw cells are activated antidromically the length of their axons should be obtained both in a more direct way and with less error

Judging from the data of Fig 4 the fibres excited with current pulses of less than 6 µA (this was the maximal strength used in this series of measurements) should lie within about 200 µm from the tip of the stimulating electrode The distance between the tip of the recording electrode and the Renshaw cell body should be at most 50—100 µm since the spikes recorded when the electrode was nearly touching the cell (for example just before its penetration) usually became indistinguishable from the noise when the electrode was withdrawn about 50 µm As the errors in defining the locations of the two electrode tips and the distance between them should not amount to more than 100—200 µm the maximal error in estimating the distance between the stimulated part of the axon and the cell body should be less than 0 5 mm

Fig 7 shows the shortest and the longest distances (black bars) from the soma at which the investigated cells were activated from within the white matter The longest distances were 11—13 mm They were found for three cells located in S1 the axons of which were found to extend to L5 Well over half of the cells were activated from distances longer than 2 mm and nearly a third from more than 5 mm However distances longer than those from which the positive results were obtained were tested only in some of the cells and not always with a sufficient number of tracks to allow conclusions on the maximal length of the axons or on the proportion of cells with long or short axons These longer distances at which activation attempts

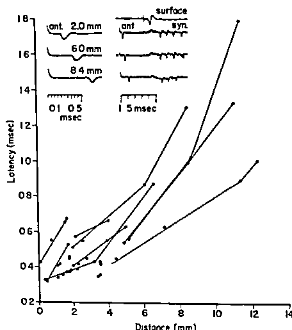


Fig 9 Conduction velocity of Renshaw cell axons The latencies (ordinate) of the antidromic spikes recorded at the somas evoked by suprathreshold stimulation in the white matter are plotted as a function of the distance (abscissa) over which the action potentials propagated Data for the same axon are connected by a line Inset records show antidromic (ant) responses from 3 stimulation sites located 2.0 6.0 and 8.4 mm from the soma and the synaptic responses following parallel stimulation of ABSm

were unsuccessful are indicated by circles for each of the cells Open circles indicate an inconclusive number of trials (the number of tracks is given to the right) while filled circles indicate that a given level was explored sufficiently to cover the whole area within which the axonal branches were likely to project (i e the motor nuclei and the region ventral to them) If the negative tests were fully reliable the maximal lengths of Renshaw cell axons would be somewhere along the dashed lines

Destination areas and target cells of single Renshaw cells A comparison of the longest distances from which single Renshaw cells were activated antidromically from within the motor nuclei the ventral funiculus or the region at a border between them (Fig 8 A—C) gives an indication of the extent of their effects on α and γ motoneurones on Ia inhibitory interneurones and other Renshaw cells The projections to motor nuclei were found mainly within one mm in either the rostral or the caudal direction although in the case of 4 cells antidromic activation was possible from longer distances up to 4 mm from their somas During tracking at more rostral levels totally at least 60 tracks crossed the motor nuclei but even with strong current pulses (up to 25 μA with a likely stimulation radius of over 500 μm) the axons were not excited On the other hand activation was achieved in over a fourth of the tracks crossing the gray matter at distances shorter than 2 mm from the somas

Projections to the areas outside the motor nuclei were found over longer distances (Fig 8 B and C) In view of this and of only rare effects on motoneurones outside a given segment (see also Eccles *et al* 1954 Hultborn *et al* 1971 d) it may be con

cluded that the bulk of long distance projections of Renshaw cells is directed to Ia inhibitory interneurones and/or other Renshaw cells

This conclusion allows a consideration of the proportion of Renshaw cells likely to terminate on both the motoneurones (α and γ) and their other target cells (Ia interneurones and other Renshaw cells) This is indicated in histograms B and C which show that a number of cells (43 %, hatched) were activated from the motor nuclei in addition to being excited by current pulses applied in the ventral funiculus and in the border region between the white matter and the motor nuclei In several cases, in which activation was obtained from sites in the white matter no attempts were made to activate the cells at distances shorter than 2 mm within which they are most likely to project to the motor nuclei Excluding these the percentage of Renshaw cells likely to reach all their target cells could be considerably larger This would speak against a functional subdivision of Renshaw cells into those affecting motoneurones only or Ia interneurones and other Renshaw cells only and corroborates a previous conclusion on multiple actions of single Renshaw cells (Hultborn *et al* 1971 c)

3 *Conduction velocity of Renshaw cell axons*

Conduction velocities were measured for fibres stimulated within the white matter but only in those cases in which the thresholds were below 4 μA They were obtained from the differences in latencies of antidromic responses induced by stimulating at slightly suprathreshold strengths the axons at different distances from the soma

The relationship between the latencies of antidromic responses and the propagation distances is shown in Fig 9 where points obtained from the same cells are connected by continuous lines Those for the shortest distances from the somas indicate maximal conduction velocities of nearly 30 m/s while those farther away correspond to conduction velocities of 6—10 m/s A decrease in conduction velocity occurred within a much shorter distance from the soma than in the case of the Ia inhibitory interneurones (Jankowska and Roberts 1972 a) indicating a greater number of early collaterals in agreement with the other findings on the projections of Renshaw cells Because of the axonal branching it is difficult to estimate the maximal conduction velocity of the initial part of the stem axons However it is noteworthy that the values around 30—40 m/s indicate external diameters of about 5—7 μm which is close to the observed diameters of Renshaw cell axons (E Jankowska and S Lindstrom to be published)

The rapid decrease in conduction velocity as the propagation distance becomes longer would considerably delay the effects of the Renshaw cells on their target cells located farther away The conduction times from Renshaw cell somas to cells located at the same rostrocaudal level 2—3 mm rostrally and 10 mm rostrally are estimated to be of the order of *0 1—0 2 0 5 and 1 0 ms respectively* In these estimates 0 2 ms were subtracted from the measured latencies of antidromic responses to allow for the latent period of excitation of fibres by electrical stimuli

For axonal branches within motor nuclei the conduction velocity could not be measured as it was impossible to follow a single branch over a sufficiently long distance. Nevertheless the latencies of antidromic responses evoked by stimuli applied in motor nuclei are clearly longer than those of the responses evoked by stimulation in the white matter at approximately similar distances (see Fig. 6). It follows that the impulses in collateral of Renshaw cell axons must propagate with considerably lower velocity than in the main branches. Thus the 0.1—0.2 ms estimate for conduction time to target cells located at the same segmental level (see above) would be valid only for those inhibited by the shortest collaterals.

Synaptic excitation of Renshaw cells by pulses applied through the microelectrode was evoked only when its tip was not farther than about 2 mm from the Renshaw cell body (Fig. 8 E) (*cf.* Ryall *et al.* 1971). Since no attempt was made to find the lowest threshold sites the relatively strong current pulses (up to 20—25 μA) were used which could have an effective radius of at least 200—300 μm. Therefore the distances over which motor axon collaterals appeared to project are estimated to be 2.0—2.5 mm. Their total length should not be much longer as the effective stimulus pulses were most often applied close to the border of the ventral horn, that is, where the motor axons were leaving it and before they started their medio-caudal course across the ventral funiculus. Moreover the trajectory of these axons within the gray matter should be rather short because in most cases they originated in PBSt and ABSm motor nuclei which are located in the ventral part of the ventral horn. The estimated lengths of motor axon collaterals are shorter than the maximum values reported by Scheibel and Scheibel (1966).

The conduction velocity along those collaterals was calculated for synaptic responses evoked from at least 1 mm distance from the Renshaw cell somas. Assuming the shortest possible trajectory of the collaterals over the measured distances, a 0.2 ms latent period for the generation of spike potentials in the motor axons, a synaptic delay of 0.3 ms (*cf.* Jankowska and Roberts 1971 a, b) and 0.2 ms between the onset of the EPSP and the generation of the spike, values were obtained ranging from 2 to 9 m/s.

Discussion

Originally a discrepancy seemed to exist between the postulated properties of Renshaw cells and the morphological description of cells found by anatomists in the ventral horn of the spinal cord. This discrepancy issued mainly from a misinterpretation of the earliest physiological findings on Renshaw cells. The facts that they inhibited primarily motoneurones in their closest vicinity (Eccles *et al.* 1954) and that until recently no other cells but motoneurones were known to be inhibited by Renshaw cells were taken to indicate that their axons should be confined to the gray matter and that Renshaw cells are probably Golgi type II neurones (Eccles, Fatt and Landgren 1956, Scheibel and Scheibel 1964, 1966). As the existence of such neurones had no support in anatomical studies (Ramon y Cajal 1909, Testa 1964, Scheibel and Scheibel 1964, 1966, 1969, 1971) 2 alternative solutions to the problem were presented: (1) to deny the existence of Renshaw cells (Scheibel and Scheibel 1964, *cf.* also Erulkar *et al.* 1968, Weight 1968) and (2) to consider them funicular cells (Szentagothai 1967, Scheibel and Scheibel 1966, 1971, Hultborn *et al.* 1971 b, Ryall *et al.* 1971, Willis 1971). In view of all the physiological evidence (see Eccles 1969) and the morphological identification of Renshaw cells by intracellular staining with Procion Yellow (Jankowska and Lindstrom 1971, van Keulen 1971) the first alternative is no longer tenable. The second alternative received its full support in the present study.

In this context some remarks should be made on the relative contributions of physiological and anatomical observations in revealing the neuronal substrates of various phenomena There is no doubt that neural circuity based on physiological observations becomes more plausible if its postulated elements or connexions are disclosed and identified morphologically However the mere absence of such morphological support or failure to find the required elements is not sufficient to invalidate conclusions based on physiological observations In particular, the inhibitory processes seem to be known sufficiently well nowadays to differentiate between direct effects of some fibre systems and effects mediated by interposed neurons—pre or postsynaptically Therefore if the morphological techniques fail to reveal the postulatd interneurones the limitations of these techniques must be considered first and they must be combined with physiological methods

On the basis of indirect evidence Ryall Piercey and Polosa (1971) concluded that the distances over which Renshaw cells project may be at least five mm In the present study it was possible to excite axons of single Renshaw cells at even longer distances more than 12 mm away from their somas and show that they may extend over 2—3 spinal segments These axons were found to project within both the ventral horn and the white matter In fact nearly all cells could be activated by current pulses applied in the ventral funiculus giving direct proof that Renshaw cells are funicular cells (*cf* also Jankowska and Lindstrom 1971) The observations on which this conclusion is based were of course limited to those cases in which the tip of the stimulating electrode was at a reasonable distance from the motor nuclei and in which the effective radius of the applied current was less than this distance

Generally it appears that recurrent effects are mediated by fairly short collaterals of motor axons terminating on Renshaw cells with relatively long axons Within 2—4 mm from their somas they may terminate on all four groups of their target cells (*α* and motoneurones interneurones mediating Ia reciprocal inhibition and other Renshaw cells) At longer distances however they would be more likely to project to Renshaw cells and/or the Ia interneurones than to motoneurones This conclusion is based both on previous observations that recurrent inhibition of motoneurones is evoked primarily (Eccles *et al* 1954) though not exclusively (Hultborn *et al* 1971 d), by Renshaw cells located in the same segment and by the failure to active Renshaw cells axons from within the motor nuclei further away This failure has however to be considered with all the reservations for the negative results especially that the sample of Renshaw cells investigated in this study was not selected from the point of view of their input and their longest expected projections onto motoneurones *A differentiation between long distance projections to Ia inhibitory* interneurones and to Renshaw cells is not possible on the basis of the present material since both these groups of cells are located in neighbouring areas However other observations seem to indicate that the long axonal branches of Renshaw cells terminate to a greater extent on other Renshaw cells than on Ia interneurones It has been shown that Ia inhibitory interneurones excited by the same group I muscle spindle afferents as a given group of motoneurones are located close to the latter

(as a rule in the same segments although not fully overlapping with the extent of the motor nuclei Hultborn *et al* 1971 b Jankowska and Lindstrom 1972) and that both are inhibited via axon collaterals of the same motor axons (Hultborn *et al* 1971 c) Thus the projections to these interneurones should in principle mirror those to the motoneurones However not much is known about the origin of recurrent inhibition of interneurones with a wider Ia convergence (Hultborn *et al* 1971 b Hultborn and Udo 1972) which may be evoked by larger populations of Renshaw cells The postulated long distance projections between Renshaw cells on the other hand will need experimental verification since they have been shown only over distances of about 5 mm (Ryall *et al* 1971)

The complex projections of Renshaw cells which may send their axons along a considerable length of the spinal cord and act on several types of cells are by themselves of great interest from the functional point of view They point to the Renshaw cells as to important links in spinal coordinatory mechanisms although much more material is needed to understand the pattern of their actions and their role in different reflexes

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Acta physiol scand 1973 88 215—225

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Renal Medullary Red Cell and Plasma Flow as Studied with Labelled Indicators and Internal Detection

By

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Abstract

WOLGAST M *Renal medullary red cell and plasma flow as studied with labelled indicators and internal detection* Acta physiol scand 1973 *88* 215—225

Regional renal medullary red cell and plasma flow has been investigated on 8 dogs using ^{32}P labelled red cells and plasma and detection with small needle shaped semiconductor detectors put into the parenchyma. The transit times were determined by dividing the area with the height of the indicator dilution curves obtained after slug injections of alternatively labelled red cells and labelled plasma into the renal artery Regional red cell volume was obtained from intrarenal equilibrium activity The plasma volumes relative to the red cell volumes was determined by comparing the areas of the two indicator-dilution curves Whole blood flow was then estimated at 2 ml/min and g tissue in the outer zone continuously decreasing to values around 0 5 ml/min and g tissue in the inner parts of the inner zone The hematocrit in vasa recta blood (denoting the ratio between the flow of red cells to the flow of whole blood) was in the outer zone 85 % of that in arterial blood successively decreasing to about 50 % in the inner parts of the renal medulla.

With the introduction of the counter current theory for the urine concentration mechanism intrarenal circulatory parameters began to receive serious attention—the purpose of which was to investigate the role of the medullary circulation in this process The first method developed which allowed for the determination of medullary blood flow was presented by Kramer *et al* (1960) In this method Evans blue labelled plasma was injected into the renal artery and detected by small photocells placed along the pelvic surface of the medulla The plasma flow was calculated from the transit time of the indicator and from known regional volumes of plasma and red cells Another approach was offered by Lilienfield *et al* (1961) using the accumulation rate of labelled albumin as an index of medullary plasma flow The aim of both of these methods was to study the absolute blood flow as expressed in ml/min and g tissue in contrast to other methods in which the transport of inert diffusible indicators (Thorburn *et al* 1963 Aukland and Berliner 1964 Schieve *et al* 1959 Sadler and Tuttle 1963 and Grangsjo *et al* 1966) or the extraction of Rb^{86} (Harsing and Pelley 1965) are used as an index of effective regional blood flow The result

from each of the previously mentioned investigations show a very low medullary blood perfusion in contrast to a high cortical blood flow even though the values obtained differ considerably probably due to technical and analytical difficulties inherent in all the methods tried

In a previous investigation (Wolgast 1968) a method was presented in which labelled red cells were injected intra arterially and detected by small needle shaped semi conductor detectors put into the renal parenchyma allowing the determination of regional red cell flow The present investigation studies both the red cell and the plasma flow in order to obtain more precise data on the blood flow in different parts of the renal medulla

Material and Methods

The experiments were carried out on 8 healthy dogs (schafer and harrier) of both sexes weighing 17—28 kg Anesthesia was induced by Penthotal sodium® (Abbot lab LTD Great Britain) 10 mg/kg followed by chloralos in saline (Chloralos puriss E Merck Darmstadt West Germany) in a dose of 50 mg/kg initially and then continued by smaller additional doses in order to keep the anesthesia as even as possible A tracheal tube was inserted to ensure free airways Catheters were placed in the femoral artery for blood pressure recording and blood sampling and into the cubital vein for infusions Via a flank incision and careful retroperitoneal dissection the left renal hilus region was exposed Silicon tubes were then introduced into the renal vein (via the spermatic or ovarian vein) for blood sampling and recirculation studies and into the left urether for continuous urine collection To make the intra arterial injections of labelled red cells and plasma a mandrin supplied nylon tube (O.D 0 75 mm) was introduced into a lumbar artery situated just opposite to the hilus region and was then passed through the aorta into the renal artery With this technique no free dissection of the renal artery or the hilus region was necessary The needle detectors were introduced into the renal parenchyma perpendicularly to the kidney surface and with their tips oriented towards the papilla. Usually two needle detectors were used—one with 3 separate elements and the other with one

The associated electronics consisted of a three channel analyzing device (AB Atomenergi Sweden) in which the signals from the detector were amplified in a charge sensitive pre amplifier (mod 4650 C) and main amplifier (mod 4631 B) After lower level discrimination (mod 4106 A) the counting rate was determined by a rate meter (mod 4603 D) and recorded on an ultraviolet light recorder (Ultralette mod 2120 ABEM Stockholm Sweden)

The stability and linearity of the detecting system have been tested and found to have a negligible drift during a 5 h period and a neglible coincidence loss in the actual range of counting rates The efficiency of the detectors with a discriminator level corresponding to about 50 eV (background then 1—[illegible] cps) is in the order of 5000 cpm if the detectors are submerged in a saline solution having a concentration of 1 µCi/ml of P^{32}

The monitored volume has been determined by moving a point source of P^{32} into different positions in relation to the detector (For a detailed description see Wolgast 1968) The monitored volumes defined as the volumes from which [illegible]0 % of the activity will arise are visualized in Fig 1 It would seem that the monitored volume of each element constitutes an oblate spheroid with a minor axis (parallel to the long axis of the detector) of about 5 mm and with a major axis of 7 mm (About 90 % of the activity recorded from P^{32} dissolved in an infinitely large volume of saline will thus arise from this spheroid

As the indicator for red cells P^{32} labelled red cells were used For labelling [illegible] ml of red cells was mixed with about 15 mCi P^{32} phosphate in a citrate phosphate buffer solution (Mollison *et al* 1958) consisting of Tr sodium citrate 3 0 g Sodium di hydrogen phosphate 0 015 g Glucose 0 [illegible] g and water to 100 ml After 1 h at 38 C when the red cells had incorporated around 75 % of the activity the cells were washed 3 times in cold saline and re suspended in their own plasma. During the course of the experiment additional washing had to be carried out due to the loss of free P^{32} phosphate from the cells As the plasma indicator P^{32} labelled chromic phosphate particles having a diameter of 100—500 Å were used (CEA CEN Sorin Italy)

The experiments were carried out by making slug injections (0 3—1 2 ml) of alternatively labelled red cells and plasma into the renal artery The indicator dilution curves obtained from the detectors were then recorded for three minutes or more The recirculation part of the curves was checked by injecting the indicators into the renal vein

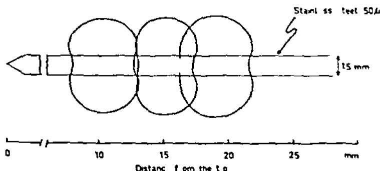


Fig 1 Monitored volumes of the 3-element detector used in the present investigation in the case of ^{32}P as the tracer label If the detector is submerged in a infinitely large volume of a ^{32}P solution about 90 of the total activity recorded will arise from tracer located within the volume shown in the figure

The regional red cell volume was determined from the intrarenal equilibrium activity attained approximately 10 min after the red cell injection and from the equilibrium activity in the blood withdrawn at that time The plasma volume was determined from the indicator dilution curves as will be described Clearance of Creatinin and PAH was made t regular intervals together with determinations of total renal plasma flow b the PAH-extraction method

Calculations

The flow of an intravascular indicator (e g red cells and plasma) in the different regions of the kidney is calculated by the general formula

$$F_i = V_i/t_i \qquad (1)$$

where F is the flow V the volume of distribution of the indicator within the parenchyma, and t the mean transit time

Regional volumes In this investigation the regional red cell volume was determined as the ratio between the equilibrium activity in the tissue and the activity in pure red cells in a blood sample drawn at the same time The red cell volume was then expressed as the per cent of the tissue volume

The regional plasma volume determination s more complicated than that for red cells since the chromic phosphate particles used will be rapidly removed from the circulation preferentially by the liver and a stable equilibrium activity is thus unobtainable Furthermore some particles will leak out into the extravascular pace in the kidney and will thus add to the volume determined The plasma volume was therefore determined from indicator dilution curves as follows

The area, A obtained during a single circulation is governed by the equation

$$A_i = \frac{M_i \ \bar{E} \ V_i}{F_{tot\,i}} \quad \text{(Wolgast 1968)} \qquad ($$

where M is the amount of indicator injected E, the efficiency for the detector V_i the indicator volume of distribution and F_{tot} the flow of the actual substance where the indicator is injected (i in the case of plasma the total renal plasma flow) The ratio areas obtained from red cell and plasma indicator dilution curves is then

$$\frac{A_{rc}}{A_p} = \frac{V_{rc} \ M_{rc} \ \bar{E} \ F_{tot\,p}}{V_p \ M_p \ \bar{E} \ F_{tot\,c}} = \frac{V_{rc} \ M_{rc} \ (F}{V_p \ M_p \ H}$$

from each of the previously mentioned investigations show a very low medullary blood perfusion in contrast to a high cortical blood flow even though the values obtained differ considerably, probably due to technical and analytical difficulties inherent in all the methods tried

In a previous investigation (Wolgast, 1968), a method was presented in which labelled red cells were injected intra arterially and detected by small needle shaped semi conductor detectors put into the renal parenchyma allowing the determination of regional red cell flow The present investigation studies both the red cell and the plasma flow in order to obtain more precise data on the blood flow in different parts of the renal medulla

Material and Methods

The experiments were carried out on 8 healthy dogs (schafer and harrier) of both sexes weighing 17—28 kg Anesthesia was induced by Penthotal sodium® (Abbot lab LTD Great Britain) 10 mg/kg followed by chloralos in saline (Chloralos puriss E Merck Darmstadt West Germany) in a dose of 50 mg/kg initially and then continued by smaller additional doses in order to keep the anesthesia as even as possible A tracheal tube was inserted to ensure free airways Catheters were placed in the femoral artery for blood pressure recording and blood sampling and into the cubital vein for infusions Via a flank incision and careful retroperitoneal dissection the left renal hilus region was exposed Silicon tubes were then introduced into the renal vein (via the spermatic or ovarian vein) for blood sampling and recirculation studies and into the left urether for continuous urine collection To make the intra arterial injections of labelled red cells and plasma a mandrin supplied nylon tube (O D 0 75 mm) was introduced into a lumbar artery situated just opposite to the hilus region and was then passed through the aorta into the renal artery With this technique no free dissection of the renal artery or the hilus region was necessary The needle detectors were introduced into the renal parenchyma perpendicularly to the kidney surface and with their tips oriented towards the papilla Usually two needle detectors were used—one with 3 separate elements and the other with one

The associated electronics consisted of a three channel analyzing device (AB Atomenergi Sweden) in which the signal from the detector were amplified in a charge-sensitive pre amplifier (mod 4650 C) and main amplifier (mod 4631 B) After lower level discrimination (mod 410[illegible] A) the counting rate was determined by a rate meter (mod 4603 D) and recorded on an ultraviolet light recorder (Ultralette mod 2120 ABEM Stockholm Sweden)

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The monitored volume has been determined by moving a point source of I^{32} into different positions in relation to the detector (For a detailed description see Wolgast 1968) The monitored volumes defined as the volumes from which 90 % of the activity will arise are visualized in Fig 1 It would appear that the monitored volume of each element constitutes an oblate spheroid with a minor axis (parallel to the long axis of the detector) of about 5 mm and with a major axis of 7 mm About 90 % of the activity recorded from I^{32} dissolved in an infinitely large volume of fluid will thus arise from this spheroid

As the indicator for red cells I^{32} labelled red cells were used For labelling 2 ml of red cells was mixed with about 15 μCi $I^{3[illegible]}$ phosphate in a citrate phosphate buffer solution ([illegible] *et al* 1958) consisting of Trisodium citrate 3 0 g Sodium dihydrogen phosphate 0 015 g Glucose 0 2 g and water to 100 ml After 1 h at 38 C when the red cells had incorporated around 75 % of the activity the cells were washed 3 times in cold saline and resuspended in their own plasma During the course of the experiment additional washing had to be carried out due to the loss of free I^{32} phosphate from the cells As the plasma indicator $I^{[illegible]2}$ labelled chromic phosphate particles having a diameter of 100—500 Å were used (CEA CEN Sorin Italy)

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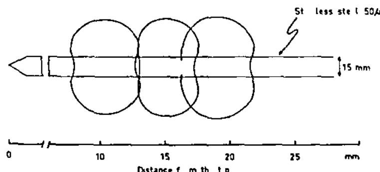


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Calculations

The flow of an intravascular indicator (*e g* red cells and plasma) in the different regions of the kidney is calculated by the general formula

$$F_i = V_i / \bar{t}_i \tag{I}$$

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The area, A_i obtained during a single circulation is governed by the equation

$$A_i = \frac{M_i \; \bar{E} \; V_i}{F_{tot\,i}} \quad \text{(Wolgast 1968)} \tag{II}$$

where M_i is the amount of indicator injected $\bar{E}$ the efficiency for the detector V_i the indicator volume of distribution and $F_{tot\,i}$ the flow of the actual substance where the indicator is injected (*i e* in the case of plasma the total renal plasma flow) The ratio between the areas obtained from red cell and plasma indicator dilution curves is then

$$\frac{A_{rc}}{A_p} = \frac{V_{rc} \; M_{rc} \; \bar{E} \; F_{tot\,p}}{V_p \; M_p \; \bar{E} \; F_{tot}} = \frac{V_{rc} \; M_{rc} \; (1-HCT)}{V_p \; M_p \; HCT} \tag{III}$$

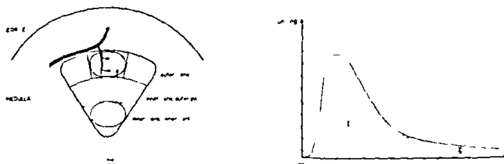


Fig 2 Monitored region of a detector placed in the middle of the outer zone and the pathway of the blood supplying this area and the corresponding indicator dilution curves as obtained *during a single circulation Part I of this curve is generated by activity passing in the* outer medullary capillary system and activity bound for inner medullary capillary systems passing through the outer medulla in descendent vasa recta. Part II is generated by activity passing in ascending vasa recta draining the inner medullary vascular system

where the subscripts rc and p stand for red cells and plasma, respectively and HCT the systemic hematocrit The efficiency factor $\mathfrak{E}$ is in the actual case the same as the same isotope has been used as the labelling substance By measuring the areas (dim.counts) of the indicator dilution curves the amount injected and the arterial hematocrit the ratio between red cell volume and plasma volume can thus be determined

Mean Transit Time For the mean transit time determination the recirculation part (as determined from the injections into the renal vein) was first subtracted from the total curve in order to get the indicator-dilution curve during a single circulation This curve could then where the medullary recordings are concerned be divided into two parts by lin log extrapolation of the first rapid decay of the curve (Fig 2) The first and major part (averaging 75 of the total curve) is assumed to be generated by a) the indicator transversing in a the capillary loops within the monitored volume and their corresponding descending and ascending vasa recta, and from b) activity passing in descending vasa recta bound for capillaries situated below the monitored volume The second part of the curve is then generated by the indicator passing in those ascending vasa recta that drain capillaries situated *below the monitored* volume This latter part is called the internal re-circulation part The mean transit time is calculated from the first and major part of the curve in several ways (Wolgast 1968) However the mean transit time obtained by dividing the area of the curve by its height is presented in this paper This time then refers to the mean time it takes for red cells and plasma respectively to pass through the monitored volume Strictly speaking however since the time is calculated from only approximately 75 of the total curve the time will reflect the passage through a volume which comprises 75 of the total volume of distribution (Equation 3) of the indicator However taking into account the overall accuracy of the method and the fact that the regional red cell volume determination will represent an underestimation moreso than in the circumvent case due to the non perfused zone around the detectors) no correction was made For a more detailed theoretical discussion reference is made to Wolgast 1968 and to Wolgast and Lauber 1962

Results

The blood pressures in this series varied between 110 and 125 mm Hg which is considered to be normal for this type of anesthesia The hematocrit averaged 0 37 (range 0 32—0 43) thus somewhat less than normal This was probably due to the fact that chloralos was administrated in relatively large amounts of fluid (20—40 ml/h) The renal blood flow was estimated at 3 3 ml min and gram tissue (2 8—4 0 ml/min) The left urin flow averaged 0 5 ml min and the osmolality 890 mO m/kg (607—

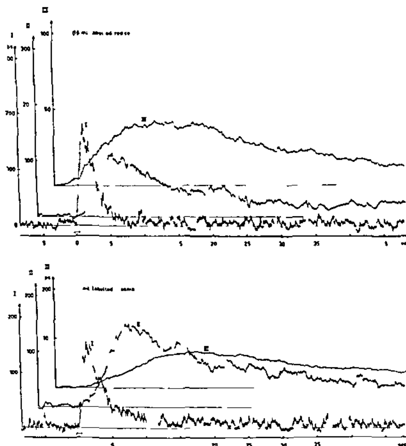


Fig 3 Cortical (I) outer medullary (II) and inner medullary (III) indicator-dilution curves obtained after the injection of labelled red cells (upper panel) and labelled plasma (lower panel)

1215 mOsm/kg) Fig 3 shows two sets of indicator dilution curves obtained after the injection of 0 6 ml of blood with labelled red cell and after 0 8 ml of labelled plasma respectively It is clearly evident that the cortical recordings (I) are very rapid in contrast to the more extended outer medullary recordings (II) and the very slow dilution curves obtained from a detector positioned in the inner zone about 9 mm from the cortico medullary border (III) It is furthermore noteworthy that the plasma transit times is only slightly longer than the red cells transit times in spite of the fact that the regional plasma volumes are much larger than the red cell volumes especially in the medullary regions In Fig 4 the transit times for red cell and plasma in the different regions of the kidney are summarized In the cortical parenchyma the transit time for red cells averaged 2 9 s and for plasma 3 2 s In the medulla the mean transit times were found to increase with increasing distance from the cortico-medullary border reaching values around 1 min near the pap

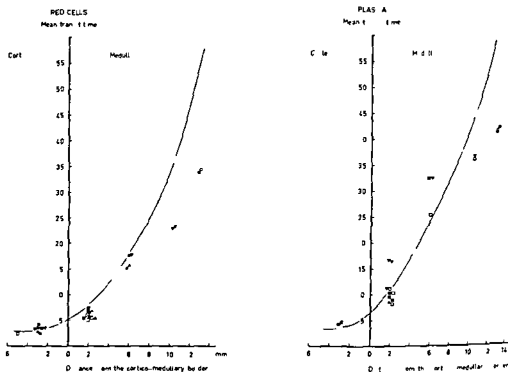


Fig 4 Red cell and plasma transit time as related to the distance from the cortico-medullary border Different symbols refer to different experiments

up The plasma transit times are only moderately longer than the corresponding red cell transit times

In Table I the mean values from two to four determinations of the transit times the regional volumes and the corresponding flows for red cells and plasma in the individual experiments are shown In Fig 5 the red cell volume and the hematocrit (denoting the ratio between the volume of red cells and the volume of whole blood) are plotted against the distance from the corticomedullary border in Fig 6 the red cell flow the whole blood flow and the hematocrit now denoting the ratio between the flow of red cells and the flow of whole blood The latter hematocrit values are expressed in per cent of the central arterial hematocrit The values for the different parameters refers to the mean value within the monitored volume thus to a segment having an extension of approximately 5 mm in the striatal direction of the parenchyma (see Methods)

In the middle of the approximately 5 mm thick outer zone (see Fig 6) the red cell flow was estimated at about 0 6 ml min and gram tissue and the corresponding blood flow to 1 8 ml/min The hematocrit in inflowing blood then constituted some 85 % of the arterial hematocrit In the inner medulla the red cell flow was 0 12 ml/min and gram in the outer 5 mm thick part successively decreasing to values around 0 05

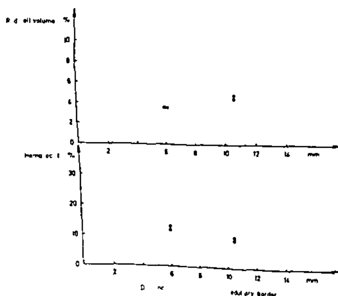


Fig 5 Regional medullary red cell volume expressed in per cent of tissue volume and the corresponding hematocrit values denoting the ratio between the intravascular red cell volume and the whole blood volume

TABLE I Data on the circulation parameters in the renal medulla

Exp no	Detector position mm from CM border	Mean t red cells seconds	Red cell volume	Red cell flow ml/min × g	Mean t plasma seconds	Plasma volume	Plasma flow ml/min × g	Hematocrit of arterial	Blood flow ml/min × g
	2	5.8	5.2	0.54	9.9	16.5	1.00	101	1.51
1	6	14.3	3.5	0.15	28.1	40.2	0.86	43	1.01
	10.5	19.4	3.2	0.10	36.4	42.5	0.70	35	0.80
	5	13.3	3.3	0.15	11.2	17.3	0.93	41	1.08
2	9	35.5	2.7	0.05	20.7	19.8	0.57	24	0.62
	13.5	51.0	3.9	0.05	26.2	17.8	0.41	29	0.45
	4.5	9.7	5.0	0.31	17.7	30.1	1.02	63	1.33
3	8.5	16.7	5.3	0.19	29.4	61.0	1.24	38	1.33
	13.0	31.7	5.3	0.10	41.3	83.0	0.95	27	1.02
4	4.5	16.7	3.1	0.11	27.7	29.3	0.48	59	0.59
	13.0	36.8	3.9	0.06	43.2	24.0	0.33	50	0.40
	2	6.0	10.4	1.04	8.2	23.1	1.69	88	2.73
5	6	18	7.4	0.24	34.9	38.8	0.67	63	0.91
	10.5	43.9	4.7	0.06	54.0	31.4	0.35	35	0.41
	[illegible]	6.6	8.7	0.79	10.5	22.3	1.27	100	2.07
6	6	28.3	4.8	0.10	39.6	35.2	0.53	42	0.64
	10.5	33.4	4.4	0.08	48.3	44.4	0.55	34	0.63
	1	13.8	4.6	0.20	13.1	11.2	0.51	70	0.71
7	5	27.5	7.4	0.16	37.4	31.5	0.50	60	0.67
	9.5	52.8	6.1	0.69	46.8	18.3	0.24	58	0.30
		10.4	—	—	−15.4	—	—	—	
8	6	17.3	3.3	0.11	33.7	27.1	0.39	58	0.51
	10.5	23.2	1.8	0.05	35.6	16.?	[illegible]	[illegible]	.5?

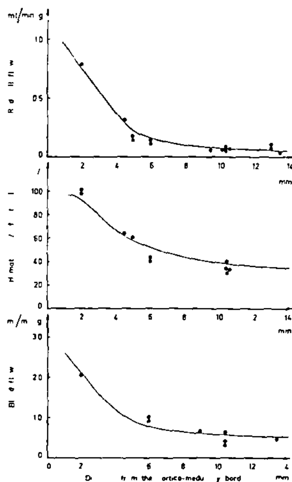


Fig 6 Medullary red cell flow whole blood flow and the hematocrit as expressed in per cent of the arterial hematocrit

ml/min in the inner parts of the inner zone The whole blood flow was there comparatively larger estimated at about 0 7 ml/min and 0 5 ml/min and gram in the outer and inner parts of the inner zone respectively The hematocrit in inflowing blood was then only half that of arterial blood or less This condition with a comparatively high plasma flow was concommitant (though not by necessity) with high plasma volumes in the medulla especially where the inner medulla is concerned The volumes averaged 19 % 29 % and 29 % in the outer zone the outer 5 mm thick part of the inner zone and the inner part of the inner zone respectively (the values from expt no 3 were excluded in the figures given) It should to be pointed out however that the figures refer to the volume of distribution of the indicator rather than true volumes

The cortical circulation has not been invesigated with respect to its red cell volume Assuming the figure of 7 % found in a subsequent investigation with a modified way of inserting the probes the red cell flow was calculated at 1 5 ml/min

and gram tissue and the whole blood flow at 4 1 ml/min utilizing the hematocrit of 0 37 found in this series The hematocrit in inflowing blood calculated from four cortical indicator dilution curves in the same way as for the medullary curves gave here a value of 0 34 thus in reasonably good accordance with the value of 0 37 valid for femoral artery blood

Discussion

In the present investigation the flow of red cells and plasma are calculated from the mean transit times and the regional volume and there may obviously be errors in both of these determinations The trauma from inserting the probe results in a damaged zone around the detector surface it also can cause a more generalized affection of the circulatory conditions within the whole of the monitored volume In the cortical parenchyma which is fragile and constitutes a highly vascularized tissue bleedings often occurred whereas in the medulla bleedings were rarely visible The red cell determinations in the cortex then result in an underestimation due to the indicators inaccessibility to the damaged blood vessels If the detector (this was performed in a later investigation) is introduced into a freely draining preformed channel the damaged zone can be markedly reduced and more accurate red cell volumes (7—8 %) can be obtained The mean transit time through the monitored volume seems however to be unaffected by the trauma as guided by the good fit over a large range between on one side the mean cortical red cell flow calculated from total renal blood flow (electromagnetic flowmeter) the systemic hematocrit and the weight of the cortical parenchyma and on the other the regional cortical red cell flow as calculated from the actual transit times obtained and a red cell volume of 8 % the latter figure was derived from data on this parameter in the literature (Aukland and Wolgast 1968)

In the medulla where the damaged zone is almost invisible the red cell determinations seem to be accurate and also correlate well with those of other investigators (Lilienfield *et al* 1958 Emery *et al* 1959)

The plasma volume was not calculated from equilibrium data in the conventional way but from the area of the first rapid component of the curve The calculated volume then represents the volume of rapidly flowing plasma particles thus an intravascular volume The values obtained are astonishingly high even though they are in good accordance with earlier data (Emery 1959 Ulfendahl 1962) The volume refers however not to the real volume but to the volume of distribution of the indicator If for instance the protein concentration in vasa recta blood is higher than in systemic blood as has been found both in micropuncture studies (Thurau *et al* 1960 Wilde *et al* 1963) and microspectrophotometrically (Wilde and Vorburger 1967) the true volumes will be proportionately less than the values given

The mean transit time in the present investigation has been calculated by dividing the area of the curve by its peak activity—a time which theoretically would represent the mean time it takes for indicator particles to pass through the monitored volume

This mode of analysis will not inevitably give the true mean transit time (not even from a theoretical point of view) but the discrepancy from the true value is probably small as has been shown in a previous investigation (Wolgast 1968)

The finding of comparatively high plasma volumes in relation to the red cell volumes but roughly the same transit time means that the hematocrit (denoting the flow ratio) is low The relevance of the hematocrit values found is strongly supported by the fact that the calculated hematocrit in blood flowing to the cortical parenchyma was essentially the same as that directly measured in the femoral artery blood These values should agree since the cortical parenchyma receives almost all renal artery blood and that should have the same hematocrit as the femoral artery blood These findings in the present investigation supports the idea of a plasma skimming in the juxtamedullary glomeruli as proposed by Pappenheimer and Kinter in 1956 Their theory would however predict the same hematocrit in all parts of the medulla The proportionally more pronounced reduction found in the inner zone would then in fact point to a red cell skimming—*i e* the red cells should short circuit between the vasa recta limbs this would mean that the crenated cells in the medullary circulation should behave differently from the intact red cells

The blood flow values obtained point to a relatively high blood flow in the outer medulla of nearly 2 ml/min and gram tissue whereas in the inner zone it is in the order of 0 5 ml/min$\times$g not very different from the blood flow within the brain for instance Assuming that the outer and inner medulla constitute 20 % and 10 % respectively of the total kidney weight (von Mollendorf and Schroder 1930) approximately 10 % of the total renal blood flow is bound for the medulla as a whole and 1—2 % is for the inner medulla The flow is here expressed as ml/min which in fact requires some comments because the figures does not by necessity refers to pure intravascular flow of *fluid* If the plasma particles are concentrated in the intravascular space by a certain factor then the true intravascular fluid will be obtained by dividing the estimated flow with this factor—the rest of the plasma inflow pass through extravascular shortcircuiting routes Some of the plasma water will thus shortcircuite between the vasa recta limbs The main driving force for this water shortcircuite will then probably be the hydrostatic pressure difference between descending and ascending vasa recta the high osmolality of the renal medulla will contribute less since the reflexion coefficient for sodium chloride and urea—the main sources of the medullary hypertonicity—probably approach zero (Ullrich *et al* 1961)

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Proximal Luminal Diameters and Cell Volume in Rats Anesthetized with Inactin and Amytal

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Abstract

Elmer M L O Kristensen and P P Leyssac *Proximal luminal diameters and cell volume in rats anesthetized with inactin and amytal* Acta physiol scand 1973 88 226—233

Luminal diameters and cellular area of cross sectioned superficial proximal convolutions were measured in freeze-dryed cryo sections of normal non-diuretic rat kidneys instantly frozen with liquid nitrogen at the end of clearance and micropuncture experiments Two series were investigated in one series rats were anesthetized with the thiobarbiturate Inactin in the other series the oxybarbiturate Amytal was used as anesthetic all other factors were equal In rats anesthetized with Amytal proximal luminal radius was about 14 μm independent of the inulin clearance (C_{IN}) over a range between 0.57 and 1 [illegible] ml min^{-1} g KW^{-1} Proximal cellular volume as estimated from the cross sectional area was also independent of the clearance value In the Inactin series proximal luminal radius correlated with C_{IN} ($p \sim 0.018$) The luminal radius was depressed from a value of 14.5 μm at a C_{IN} about 1.40 ml min^{-1} g KW^{-1} to 12.0 μm at a C_{IN} of about 0.50 ml min^{-1} g KW^{-1} a value significantly less than that of the Amytal series Although the cross sectional cellular area tended to increase at low clearance values in the Inactin series the increase was not statistically significant The data do not exclude however the existence of an inverse relationship between cellular volume and C_{IN}

Considerable discrepancy on results in tubular function have been obtained by the various groups studying so called normal non diuretic rats The discrepancies have contributed to the disagreement in interpretation of the fact that proximal reabsorption rate and glomerular filtration rate vary in parallel within the spontaneous range of variation For several years variations in proximal luminal radius were in the focus of interest as a possible causal factor for the variations in reabsorption rate (Gertz *et al* 1965) an idea now generally abandoned However it remains a fact that several investigators observed a co variation of the proximal luminal radius with inulin clearance (*e g* Wahl *et al* 1967 Schnermann *et al* 1968), while others were unable to find any variation in luminal diameter over the spontaneous range of clearance values (*e g* Leyssac 1963 Baines *et al* 1968)

The present study is part of an investigation aiming at a clarification of the difference(s) in experimental conditions responsible for the observed difference in functional states Parts of this investigation are presented in previous papers (Elmer *et al* 1972 Christensen *et al* 1972) It was shown that the 2 different anesthetics used the thiobarbiturate Inactin and the oxybarbiturate Amytal respectively are responsible for the differences in the 2 distinct functional states obtained Further the data indicated that proximal reabsorption rate was depressed by Inactin and that the variation in reab orption rate (and filtration rate) observed in Inactin anesthetized rats was induced by the anesthetic which somehow limits the proximal reabsorption capacity In contrast proximal reabsorption rate was higher in rats anesthetized with Amytal and independent of the serum Amytal concentration indicating that the rate of reabsorption was limited by factors other than the anesthetic (Elmer *et al* 1972) It was furthermore shown that—in rabbit gall bladders—Inactin is a more potent inhibitor of the isosmotic fluid transport than Amytal its effect was irreversible while that of Amytal was reversible (Christensen *et al* 1972)

The reciprocal of the proximal luminal occlusion time (l/OT) and of proximal transit time 1/TT) increased in direct proportion to the inulin clearance (Cl_{IN}) in the functional state obtained in Amytal anesthetized rats (state A) (Bojesen and Leyssac 1969 Elmer *et al* 1972) implying that the luminal diameter remained unchanged over the spontaneous range of variation In the state of function obtained with Inactin l/OT and l/TT were constant over the range of variation induced by the anesthetic implying a direct relationship between proximal luminal diameter and Cl_{IN}

In order to test these inferences made from the functional parameters the kidneys were snap frozen at the end of the experiment and cryo-sections obtained The present paper presents the results obtained from direct measurements of proximal luminal diameters in cryo sections of these kidneys Furthermore cellular volume was estimated in order to test whether or not it might explain the observed changes in luminal diameter

The results are quantitatively consistent with the inferences made from the functional parameters and previous observations showing a significant relationship between *proximal luminal diameter and* Cl_{IN} *in rats anesthetized with Inactin In* Amytal anesthetized rats luminal diameter was invariant with Cl_{IN} Proximal cellular volume was unrelated to proximal reabsorption rate in Amytal anesthesia under Inactin anesthesia cellular volume was approximately the same as in Amytal anesthetized rats and even though cell volume tended to increase at the lowest transport rates a significant relationship could not be demonstrated

Methods

Male SPF Spraque D wley rats weighing 240—760 g were allo ed free access to food and water prior to the experiment.

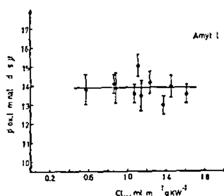


Fig 1

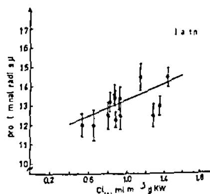


Fig 2

Fig 1 Proximal luminal radius plotted versus inulin clearance in rats anesthetized with Amytal Each point represents the mean value of 50 proximal convolutions in a kidney Bars indicate ±2 S E

Fig 2 Proximal luminal radius plotted versus inulin clearance in rats anesthetized with Inactin Each point represents the mean value of 50 proximal convolutions in a kidney Bars indicate ±2 S E

One group was anesthetized with i p injection of sodium amobarbital (sodium Amytal) 15 mg/100 g b wt supplemented when necessary during the experiment The other group was given Inactin (Promonta Hamburg) 12—25 mg/100 g b wt i p The experimental procedure was given in detail in the previous paper by Elmer *et al* (1972) Briefly the rats were placed on a thermostatically heated operating table and prepared for clearance micropuncture and occlusion time measurements After a priming dose of 15—20 mg 1 % Inulin in 0 9 % saline was infused continuously through a catheter in the jugular vein at a rate of 0 15 mg/min Urine was collected over a period of 15—30 min for clearance measurements and plasma samples were obtained before and after each clearance period Occlusion time (OT) and Lissamine green transit time (TT) were measured at the end of the clearance period as described by Leyssac (1964) and Gertz *et al* (1965) respectively Intratubular pressures (ITP) were measured by the Landis method with a Hansen capacitance pressure transducer Intratubular pressures were measured in several convolutions during each clearance period Immediately after measurements of OT and TT the left (experimental) kidney was instantly frozen in isopentane chilled with liquid nitrogen to about −160 C The time from cutting the renal pedicle to freezing was less than 1 s Cortical sections 3—4 µm thick were cut tangentially to the surface of the kidney in a cryostat Sections were cut only at the level of the superficial nephrons The sections were freeze-dried for 24 h at −25 C in close approximations to osmic acid crystals in a dessicator (Faarup 1965) thereby combining fixation with staining by osmic acid vapour The freeze-dried sections were mounted in paraffin oil under a cover glass

Photomicrographs were taken through a 10 times objective in a Reichert microscope with a Reichert Photoautomat The diapositives were projected by means of a plain mirror to a smooth white paper on the table Constant magnification was checked each day as described in detail previously (Leyssac 1965)

The selection of tubules for measurement was randomized by measuring all cross-sectioned circular proximal tubules in focus until a total of 50 convolutions had been measured in each kidney The proximal luminal diameter was measured in 2 perpendicular dimensions cellular area was measured by planimetry Each tubule was measured twice and averaged All data are presented as means ± 2 S E Calculations of proximal reabsorption rates from Cl_{IN} OT and TT are described in the previous paper (Elmer *et al* 1972)

In the statistical analysis conventional regression analysis (least squares) was used The analysis was carried out by lic techn M Larsen and stud polyt Axel af Rosenborg Dept of Medical Data Processing Centrfte County Hospital Copenhagen Denmark

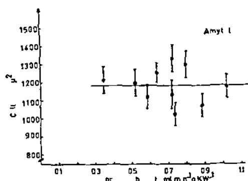


Fig 3

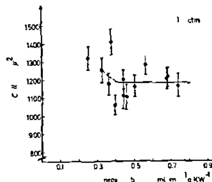


Fig 4

Fig 3 Cellular area of cross-sectioned proximal convolutions plotted versus calculated proximal reabsorption rate in rats anesthetized with Amytal Each point represents the mean value of 50 convolutions in a kidney Bars indicate ± 2 S E

Fig 4 Cellular area of cross-sectioned proximal convolutions plotted versus calculated proximal reabsorption rate in rats anesthetized with Inactin Each point represents the mean value of 50 convolutions in a kidney Bars indicate ± 2 S E

Results

In Fig 1 proximal luminal radius is plotted as a function of inulin clearance in 10 rats anesthetized with Amytal All data from these rats fulfilled the criteria defining the Amytal state A characterized by Bojesen and Leyssac (1969) The data show that the luminal radius was approximately 14 μ independent of the clearance value within the range of variation between 0 57 and 1 61 ml min^{-1} g KW^{-1} The regression line ($y = -1\,00 \times + 14\,69$ S E regress coeff 0 95 N = 10) is not significantly different from the horizontal line drawn through the mean value ($0\,3 < p < 0\,4$ two tailed test)

In 13 rats anesthetized with Inactin proximal luminal radius did vary with Cl_{IN} as evident from Fig 2 All data from these rats were typical of the Inactin state described in the previous paper (Elmer *et al* 1972) In view of the correlation between luminal radius and Cl_{IN} in rats anesthetized with Inactin observed previously (*e g* Wahl *et al* 1967 Schermann *et al* 1968) and the observed constancy of the OT under these conditions (Wahl *et al* 1967 Elmer *et al* 1972) a positive correlation is a priori expected A one tailed test shows that the regression line ($y = 1\,82 \times + 11\,29$ S E regress coeff 0 76 N = 13) differs significantly from a horizontal line ($p \sim 0\,018$) The luminal radius varied from 12 0 to 14 5 μm over the range of clearance values from 0 53 to 1 44 ml min^{-1} g KW^{-1}

Further in order to analyze whether or not the luminal radius was significantly reduced in the Inactin series as compared with the Amytal series a common regression line for all the data was calculated (N = 23) A Wilcoxon Man Whitney test indicates that the data (residuals) of the 2 series were systematically distributed

and that the proximal luminal radius of the Inactin series was significantly less than that of the Amytal series ($p \sim 0.026$)

Similar relationships as those given in Fig 1 and 2 were also obtained when luminal radius was plotted *versus calculated* proximal reabsorption rates

Proximal intratubular *hydrostatic* pressures (PITP) were measured during the clearance period in *both groups* The mean PITP was 12.8 ± 0.7 mm Hg in the Amytal state and 12.96 ± 0.9 *mm Hg in* the Inactin state The difference is not statistically significant In *neither of* the 2 groups could a significant correlation between proximal intratubular pressure and reabsorption rate be demonstrated but in the Inactin anesthetized rats there was a tendency of the high pressure range to occur at low reabsorption *rates* (and small luminal radii)

Changes in proximal *cellular* volume occurring *after luminal* occlusion have previously been demonstrated from *measurements* of the area of cross sectioned proximal tubules (Leyssac 1965) Cellular volume in the 2 groups of rats was therefore estimated *from measurements* of the cellular area of cross sectioned proximal convolutions *Fig 3* gives the *results obtained* in rats anesthetized with Amytal The data indicate that the *mean cellular* area was independent of spontaneous variations in *proximal* reabsorption rate over the entire range of values obtained The regression *line* ($y = -72.9x + 1235$ S E regress coeff 181 N = 10) is not significantly different *from* the horizontal line drawn through the mean value ($0.6 < p < 0.7$ two tailed test)

The results *obtained* in rats anesthetized with Inactin are less clear cut as apparent from Fig 4 *It might appear as if cellular* area tended to increase at reabsorption rates below 0.4 ml min^{-1} G KW^{-1} However the increase if any was small and insignificant *Above a* proximal reabsorption rate of 0.4 ml min^{-1} g KW^{-1} cross sectional *cellular* area was the same as that measured in kidneys of Amytal anesthetized rats and *independent* of the rate of net transport The regression line ($y = -131x + 1271$ S E regress coeff 188 N = 13) calculated from all the data is not significantly *different from* the horizontal line drawn through the mean value ($0.40 < p < 0.50$ two tailed test)

Discussion

The present study clarifies the reason *for the disagreement* between the groups of investigators who have *reported a relationship* between proximal luminal diameter and glomerular filtration *rate* (GFR) and those who have failed to demonstrate such a relationship *In 2 series of* rats one anesthetized with the thiobarbiturate Inactin *the other with the oxybarbiturate Amytal all* other factors *being equal* a significant relationship was *demonstrable* in the Inactin series while proximal luminal diameter was *invariant* with inulin clearance in the Amytal series These results are in complete agreement with previous reports The lack of any variation in proximal diameter over the range of spontaneous variation in GFR reported by Leyssac (1963) and Baines *et al* (1969) was obtained in rats anesthetized with

Amytal Similarly Arrizurieta Muchnik *et al* (1969) were unable to find any significant change in luminal diameter when GFR was depressed by partial clamping of the renal artery or aorta in normal expanded or uninephrectomized expanded or non diuretic rats anesthetized with the oxybarbiturate sodium pentobarbital

The absolute value of the proximal luminal radius approximately 14 μm measured in freeze dried cryo ections of the present Amytal series is also in complete agreement with previous direct measurements A proximal luminal radius of 13—14 μm in sections of instantly frozen freeze substituted kidneys was reported by Leyssac (1963) From *in vivo* photomicrographs of surface convolutions at various rates of filtration Baines *et al* (1968) measured values about 10 μm Their measurements however did not include the refractile zone present at the outer margin of the tubular lumen under exposure with incident light This refractile zone is part of the tubular lumen as demonstrated by Bojesen and Leyssac (1969) When the original photographs of Baines *et al* were remeasured including the refractile zone a luminal radius of 13—14 μm was found Finally Hegel *et al* (1967) estimated an apparent electrical radius of approximately 14 μm from measurements of the electrical resistance across the proximal tubular wall and passive voltage attenuation along the tubular axis

The previous findings that proximal luminal radius may vary as a function of the GFR in normal rats reported by Wahl *et al* (1967) Schnermann *et al* (1968) and Levy and Windhager (1968) were all obtained in rats anesthetized with Inactin The present data therefore agree with these observations and indicate that the correlation is a co variation somehow caused by the thiobarbiturate

The slope of the present regression line (Fig 2) is less steep than that presented by Wahl *et al* also it is less than that expected under conditions of invariance of the OT (and TT) as has been demonstrated in Inactin anesthetized rats (Wahl *et al* 1967 Elmer *et al* 1972) However in view of the large scatter in OT and radii for a given clearance value and the relatively few measurements in the present series our data do not permit any quantitative estimate of the actual slope of the regression line

The question then arises why the luminal diameter varies as a function of the inulin clearance in Inactin anesthetized rats as opposed to rats anesthetized with Amytal The proximal intraluminal pressure was not significantly different in the two series nor did it correlate with the clearance in either series However the hydrostatic pressure cannot be entirely ruled out as a possible cause since the pressure difference across the tubular wall is unknown in these experiments Inactin is a potent inhibitor of isosmotic fluid transport proximal reabsorption rate was depre ed and limited by the barbiturate in the pre ent Inactin series as shown by Elmer *et al* (1972) Further Andersen (1970) showed that thiobarbiturate may increase the sodium permeability of the toad bladder Thus it was possible that the proximal luminal diameter decreased with decreasing tran port rate from a normal value of about 14 μm down to 12 μm because of cellular swelling induced

by the thiobarbiturate poisoning Even though a statistically significant correlation between proximal cross sectional area and rate of reabsorption was not obtained the present data do not rule out this possibility the cellular area of cross sectioned proximal convolutions was not sufficiently well defined If the external diameter is assumed to remain constant the variance of the area measurements indicates a corresponding variance of the luminal radius exceeding a change from 14 to 12 μm A third alternative explanation should also be mentioned Rostgaard and Thuneberg (1969) and Rostgaard *et al* (1972) have presented electron microscopic evidence of a contractile filamentous system in the basal part of rat proximal tubule cells Bundles of thin filaments of dimension similar to that of actin filaments and complexing with heavy merobyosin (HMM) were found to be transversely oriented around the tubule This finding opens the possibility that the luminal diameter (or *the elastic properties of the tubular wall) may be actively adjusted by the tone of this* intracellular contractile system In this connection it should be mentioned also that Inactin in contrast to Amytal was occasionally seen to induce smooth muscle contractions in the gallbladder wall (Christensen *et al* 1972)

The finding that proximal cross sectional cellular area was independent of the rate of reabsorption in the Amytal series agrees with previous observations from our laboratory (Leyssac 1965) only the present absolute value of the cross sectional area is higher than that reported previously This difference is due to the difference in the techniques applied—freeze substitution versus freeze-drying (*cf* Faarup *et al* 1971) These latter authors argue that freeze drying combined with osmic acid fixation eliminated cell volume changes due to the preparation while alcohol substitution causes tubular shrinkage The interpretation that proximal cell volume —and thus intracellular ion and water content—is independent of the rate of net transport over the spontaneous range of variation is uninfluenced by this difference in absolute values

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Acta physiol scand 1973 88 234—247

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Effects of Insulin and Testosterone on Glycogen Synthesis and Glycogen Synthetase Activity in Rat Levator Ani Muscle

By

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Abstract

ADOLFSSON S *Effects of insulin and testosterone on glycogen synthesis and glycogen synthetase activity in rat levator ani muscle* Acta physiol scand 1973 88 234—247

The temporal relationships for hormonal stimulation of glycogen synthesis are not clarified In the present study glycogen synthesis and the activation of the rate limiting glycogen synthetase enzyme (EC 2 4 1 11) were investigated in levator ani muscles from prepubertal male rats during insulin stimulation *in vitro* for 5—240 min and testosterone stimulation *in vivo* for 4—36 h For both hormones glycogen content was gradually increased to a level of 8 5—9 0 mg/g tissue An activation of the synthetase enzyme by means of an increase of its I form was found after only 5 min of insulin stimulation The increase in I form was continuously reduced and reached control level after 150 min of incubation For testosterone the I form was steadily increased 6—16 h after injection of the hormone The incorporation of glucose ^{14}C into glycogen was increased in both studies even when the I form was no longer increased The level of glucose 6 phosphate was also measured and the results suggest an activation of the synthetase enzyme by changes in the concentration of this metabolite during hormonal stimulation It is concluded that the length of stimulation is of importance for the stimulation of muscle glycogen synthesis by insulin and testosterone

Insulin increases accumulation of glycogen in mammalian muscle cells This effect is due to an increase in uptake of glucose and enhancement of the rate of glycogen synthesis which has been demonstrated both *in vivo* and *in vitro* (for ref see Villar Palasi 1968) A similar effect has been described for testosterone in muscles involved in the sex accessory organs of the male As there is a lag period of several hours before the effect of testosterone can be revealed the influence of this hormone is usually studied *in vivo*

In characterizing the hormonal influence on glycogen synthesis the effects on the glycogen synthetase enzyme (UDP glucose α 1 4 glucan α-4 glucosyltransferase EC 2 4 1 11) should be considered since this enzyme controls the rate of glycogen synthesis (Villar Palasi and Larner 1961) The activity of the synthetase enzyme is increased when there is an increase of its I form During standard assay conditions the I form is independent of glucose 6 phosphate (glucose 6-P) whereas the other

form of the enzyme called D form is dependent on this co factor The two forms of the enzyme are interconvertible through phosphorylation dephosphorylation sequences (Friedman and Larner 1963)

An increase in the active I form of the synthetase enzyme during stimulation of glycogen synthesis was first demonstrated with insulin *in vitro* in rat diaphragm muscle after short time incubation (10 and 30 min) with the hormone (Villar Palasi and Larner 1961) Later it was also demonstrated *in vivo* in rat hind leg muscle 5—30 min after an intraperitoneal (i p) injection of the hormone (Goldberg *et al* 1967) In rat diaphragm muscle *in vivo* no obvious effect of insulin on the I form of the enzyme was found 30—240 min after an i p injection of the hormone (Sovik 1966) In a similar *in vivo* study from this laboratory an increase of the I form in the diaphragm was shown after 10 and 20 min but not after 50 min and later (Adolfsson 1972) Thus it appears from these *in vivo* studies that the length of insulin stimulation is of importance for the activation of the synthetase enzyme Therefore it is also of interest to investigate the influence of insulin *in vitro* with respect to longer periods of stimulation The present study reports the results of such an investigation performed with the isolated levator ani (LA) muscle preparation of the prepubertal male rat incubated for 5—240 min with and without insulin The LA muscle preparation was chosen since it is better suited for long time incubations than the *in vitro* preparations of the rat diaphragm (Arvill and Ahren 1966) In previous studies the glycogen synthesis in this muscle was found to be highly sensitive to insulin (Sovik and Adolfsson 1968 Adolfsson and Sovik 1968)

The LA muscle is also sensitive to testosterone In previous studies glycogen synthesis and glycogen synthetase activity in this muscle have been investigated in castrated rats after injection of testosterone (*cf* Bergamini *et al* 1969) Glycogen content increased and an increase of the I form of the enzyme was found 12 h after injection of the hormone The enzyme was not studied for shorter periods To clarify the temporal relationships in the stimulation of the glycogen synthesis by testosterone the influence of the hormone was investigated in LA muscles from non-castrated prepubertal rats 4—36 h after injection of the hormone

For both insulin and testosterone glycogen content incorporation of glucose ^{14}C into glycogen and the 2 forms of the synthetase enzyme were measured as well as the levels of glucose 6-P In addition the synthetase enzyme of the LA muscle was characterized with respect to dependence on UDP glucose and glucose 6 P

Some preliminary results have been briefly reported previously (Adolfsson and Ahren 1968 1970)

Methods

Experimental procedure

Prepubertal male Sprague Dawley rats weighing 50—60 g were used They were fed a semi synthetic diet (Gustafsson 1959) and were deprived of food for 18—20 h before sacrifice by cervical fracture In the experiments with testosterone testosterone propionate in arachis oil (25 mg/ml) was injected subcutaneously (s c) in a dose of 100 mg/kg b w Control animals received arachis oil without testosterone

LA muscle preparations were prepared as described by Arvill and Ahrén (1966) In the *in vitro* experiments the preparations were incubated in a gyratory shaking bath at 37 C in 1 ml Krebs Ringer bicarbonate buffer pH 7 4 gassed with a O_2 CO_2 mixture (95 5 v/v) equilibrated with water When present glucose was in a concentration of 2 mg/ml and D glucose ^{14}C (U) in an activity of 2 $\mu Ci/ml$ The labelled glucose (2—4 $\mu Ci/mmol$ obtained from the Radiochemical Centre Amersham England) was never added in absence of glucose Muscle were transferred to fresh buffer every 90 min during longer incubation periods

Immediately after the incubation the muscle preparation was transferred to an ice chilled filter paper soaked in 0 9 % NaCl In the *in vivo* experiments this was done immediately after sacrifice The LA muscle was thereafter rapidly dissected free from the rest of the muscles of the preparation blotted on filter paper weighed and transferred to a tube with KOH for isolation of glycogen or homogenized in ice chilled Tris buffer for synthetase assay For glucose 6 P determinations three muscles were placed against the inner walls of a tube chilled in liquid nitrogen and later homogenized in $HClO_4$

Porcine insulin 10-times recrystallized was kindly supplied by Novo Research Laboratories (Copenhagen Denmark) Lot no S23267 For each experiment it was dissolved in 3 mM HCl to give 20 IU/ml and diluted with buffer to the concentrations desired Testosterone propionate in arachis oil (Neohombreol) was obtained from N V Organon (Oss The Netherlands)

Analytical methods

Glycogen was isolated from a KOH digest of muscle by ethanol precipitations and determined with an all enzymatic method including hydrolyzation by an α 1 4-α 1 6 amylase and subsequent spectrophotometric determination of the liberated glucose by the glucose-oxidase peroxidase method (for details see Adolfsson 1972) Rabbit liver glycogen was used as standard Values are expressed in mg glycogen/g wet tissue The ^{14}C activity of the glycogen from incorporated glucose ^{14}C was measured by liquid scintillation technique using the scintillation fluid described by Bray (1960) Aliquots of the isolated glycogen dissolved in water were counted for periods sufficient to give standard errors less than 3 % As judged by external standards there were no significant variations in the degree of quenching Incorporation of glucose ^{14}C into glycogen is expressed in CPM/mg wet tissue

Glycogen synthetase activity was measured as radioactivity incorporated into glycogen from UDP glucose ^{14}C A modification of the method described by Villar Palasi *et al* (1966) was used LA muscles were homogenized in Tris buffer (1 40 w/v pH 7 8 50 mM Tris 5 mM EDTA 10 mM NaF) and the homogenate centrifuged (2 C 5000×g 30 min) Fifty µl aliquots of the supernatant were incubated for 10 min at 30 C with 50 µl reaction mixture consisting of the Tris buffer described above in addition containing (final concentrations in the assay) 10 mg/ml of glycogen 5 mM UDP glucose and 0 5 $\mu Ci/ml$ of UDP glucose ^{14}C (U) The latter compound was obtained from NEN Chemicals Boston Mass USA with a spec activity of > 200 mCi/mmol When present glucose 6-P was in a concentration of 10 mM Both in the presence and absence of glucose 6-P the synthetase reaction was linear for 5—30 min In addition the 10 min assay was linear for final extract dilutions of 1/250 to 1/30 The reaction was stopped by the addition of 0 6 ml 6 % trichloroacetic acid containing 23 mM LiBr and 1 mg/ml of glycogen After centrifugation glycogen was isolated from 0 5 ml of the supernatant by ethanol precipitations and its radioactivity measured as described above for the determinations of the incorporation of glucose ^{14}C into glycogen Enzyme activities measured in the absence and presence of 10 mM glucose 6 P are referred to as I form and (I + D) form activity respectively Values are given in units (U) or milliunits (mU) per g wet tissue (1 mU = 1 nanomol of glucose incorporated into glycogen from UDP glucose per min at 30 C The activity of the I form is also expressed in per cent of (I + D) form activity which was unchanged during all experimental conditions In some experiments enzyme activities were related to the protein concentration of the supernatant of the muscle extract instead of to the wet weight of the muscle The hormonal effects were identical for both ways of expression Protein was determined according to Lowry *et al* (1951) For the determinations hitherto described duplicate samples were analysed for each muscle

Glucose-6-P was measured spectrophotometrically on the $HClO_4$ extracts of the muscles using the appropriate enzymes (Adolfsson *et al* 1972) Values are expressed in µmol/ml intracellular fluid *In vivo* the volume of intracellular fluid constitutes 70 % of the wet weight of the LA muscle (Arvill and Ahrén 1966) and is not influenced by testosterone (Mill and Spaziani 1968) *In vitro* the intracellular volume after incubation for 30 120 and 240 min constitutes 57 55 and 53 % respectively and is not influenced by insulin (Arvill and Ahrén 1966 1967) Total tissue water was the same *in vivo* and *in vitro* These values were used in the calculations

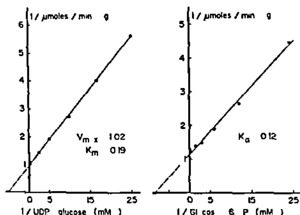


Fig 1 Lineweaver Burk plots for the dependence on the glycogen synthetase activity of the LA muscle on the concentration of UDP glucose and glucose 6 P *Left panel* Dependence upon UDP glucose in presence of 10 mM glucose 6-P Except for varying the concentration of UDP glucose standard assay conditions (see Methods) were employed *Right panel* Activation by glucose 6-P Enzyme activity was determ ned in the standard assay with various concentrations of glucose 6 P To get an indication of the activation of the D form I form activity (obtained in absence of glucose 6 P) was subtracted from the (I+D) form activity measured For the investigations presented in the two panels a centrifuged standard homogenate (25 mg tissue/ml) of muscles with low I form activity (10 %) was used The low I form was obtained by storing muscles for 6 weeks at −30 C as described by Rosell Perez *et al* (1962) Each point represents the mean of 4 determinations Calculated regression lines are shown

Statistical analyses
Mean values ± S E are given Differences between mean values were evaluated with Student s t test. For the data of Table I and V the one way analysis of variance followed by Student Newman Keul s test was used (*cf* Woolf 1968) A p value of 0 05 is considered significant in this study

Results

Kinetics of the glycogen synthetase enzyme of the LA muscle

The dependence of the synthetase enzyme for UDP glucose and glucose 6 P was characterized under standard assay conditions Fig 1 shows the data of one experiment plotted according to Lineweaver Burk Three experiments were performed In the presence of 10 mM glucose 6 P and 5 mM UDP glucose the maximal velocity of the enzyme V_{max} was 0 8—1 2 U/g wet tissue A K_m value for UDP glucose of 0 16—0 19 mM was observed The concentration of glucose 6 P giving half maximal activation K was found to be 0 12—0 15 mM

The values obtained for K_m and K_a are in good agreement with those previously reported for rat skeletal muscle (for ref see Villar Palasi *et al* 1966) The maximal enzyme activity V_{max} is about 50 % of that determined for rat skeletal muscle by similar techniques For the diaphragm muscle a V_m value of 1 7 U/g wet tissue was obtained in this laboratory The difference between the LA muscle and the diaphragm muscle is not unexpected as they represent different types of muscles The LA muscle has a high proportion of white muscle fibres (Venable 1966) whereas the diaphragm has mainly red muscle fibres (Bar and Blanchaer 1965) A higher total enzyme activity in red than in white muscle is consistent with the data of St George Stubbs and Blanchaer (1965)

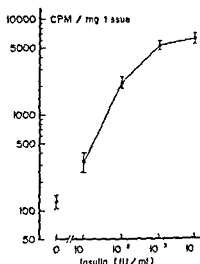


Fig 2 Dose response curve for the effect of insulin on incorporation of glucose ^{14}C into glycogen in rat LA muscle incubated for 120 min Intact LA muscle preparations were incubated in the presence of glucose (2 mg/ml) and the concentrations of insulin indicated Incorporation of glucose ^{14}C was measured as described in Methods Each point represents the m an of 4—5 muscles S E × 2 is indicated by the length of the vertical bars

Dose response relationships for the effect of insulin on glycogen synthesis and glycogen synthetase activity

Intact LA muscle preparations were incubated for 120 min in the presence of glucose 2 mg/ml and insulin 10^{4} to 10^{1} IU/ml As illustrated in Fig 2 incorporation of glucose ^{14}C into glycogen was markedly enhanced by the hormone A maximal effect was obtained with 10 IU/ml of insulin The accumulation of muscle glycogen was significant at a concentration of insulin of 10^{4} IU/ml and showed maximal values for 10 IU/ml (Table I) The influence of various concentrations of insulin on synthetase enzyme activity was determined after 15 min of incubation At this time glycogen content was the same in control and insulin stimulated muscles which is of advantage of minimizing an influence of glycogen *per se* on enzyme activity Total synthetase enzyme activity (I+D) form was not changed by insulin (Table I) A graded response of the per cent active I form of the enzyme was found when insulin was increased from 10^{3} to 10 IU/ml (Table I) As both glycogen synthesis and synthetase enzyme activity were maximally stimulated by 10 IU of insulin/ml this concentration was used in the further experiments with this hormone

Time relationships for the influence of insulin on glycogen synthesis and glycogen synthetase activity

The incorporation of glucose ^{14}C into glycogen during various lengths of incubation is shown in Table II Control muscles had a very low rate of incorporation which after 30 and 60 min of incubation was not significantly increased above zero level With insulin (10 IU/ml) incorporation was markedly enhanced during the 120 min of observation

The influence of insulin *in vitro* on glycogen content and % synthetase I form during 30—240 min is shown in Fig 3 LA muscle preparations were incubated in

TABLE I Dose response relat onship for the effect of insulin on accumulation of glycogen and activa tion of glycogen synthetase enzyme in rat LA muscle *in vitro*

	Concentration of insulin (IU/ml)				
	0	10	10	10	10⁻
Glycogen after incubat on for 120 min mg/g wet tissue	2 85±0 15	3 6? ±0 31	4 76±0 17	6 01±0 37	5 96±0 24
Synthetase enzyme after incubation for 15 min I form	23 5±2 4	—	32 2±1 5	38 2±2 0	41 8±2 9
I form mU/g wet t ssue	166±19	—	213±17	299±27	307±37
(I+D) form mU/g wet tissue	708±37	—	662 ±37	781±57	726±5?

Intact LA muscle preparations were incubated with the concentrations of insulin ndicated Glycogen was measured after 120 min and synthetase enzyme act vity after 15 m n The analyses are described in Methods There are 5–6 rats in each group Mean values±S E are g ven Clycogen was increased at the lowest insulin concentration ($p < 0 05$) Per cent I form was increased by 10⁻ IU of insulin/ml (I+D) form was unaffected by insulin For all parameters there was no difference between 10 and 10⁻ IU/ml

TABLE II Time relationship for incorporation of gluco e C into glycogen during insul n stimula tion of rat LA mu cle *in vit o*

Parameter		Incubat on time (m n)			
		15	30	60	120
Incorp of glucose C CPM/mg wet tissue	Control	33±10	76±32	47±27	107±36
	Insulin	260±47	940±130	2740±240	7730±350
Glycogen mg/g wet tissue	Control	—	3 80±0 16	4 09±0 12	4 01±0 20
	Insulin	—	4 33±0 14	5 29±0 26	7 31±0 17

Muscles were incubated for the periods ind cated in absence or pre ence of insulin 10⁻ IU/ml Me n valu s of 4 obs rvations±S E are given

the presence of glucose and transferred to new vessels with fresh medium every 90 min to minimize a decrease in the concentration of glucose and insulin during in cubation With this technique the glycogen content of the control muscles was not significantly reduced during the period investigated In the presence of insulin glycogen was continuously accumulated up to a level of 8 5—9 0 mg glycogen/g wet tissue which was reached after approximately 150 min of incubation The increase in glycogen was significant alter 30 min Concerning the syntheta e enzyme the % I form was almost doubled by insulin after 30 min and was thereafter gradually reduced with length of incubation After 150 min with insulin the % I form was no longer increased compared to controls and after 240 min there was a significant decrease Total enzyme activity (I+D) form was not affected by insulin In other

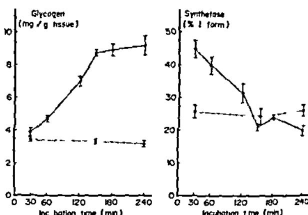


Fig 3 Time relationship for accumulation of glycogen and activation of glycogen synthetase enzyme during insulin stimulation of rat LA muscle *in vitro* Muscles were incubated in presence of glucose (2 mg/ml) for the periods indicated on the abscissas They were transferred to fresh media every 90 min The concentration of insulin was 10^{-2} IU/ml Glycogen and % synthetase I form were measured in the same experiment but not on the same muscles Compiled data from 2 experiments are shown Each point represents the mean of 4—6 observations Broken lines represent muscles incubated without insulin S E × 2 is indicated by the length of the vertical bars. For incubations of 150 min and longer there was no further increase in glycogen level and no increase in % I form compared to controls At 240 min the % I form was significantly decreased

experiments an increase in synthetase I form was observed as early as after 5 min of incubation with insulin (10 IU/ml) This effect was also seen without glucose in the incubation medium (Table III)

In order to clarify the inability of insulin to increase synthetase I form over control level after long periods of incubation (Fig 3) further experiments were performed The long incubation time *per se* was not responsible for this lack of effect An increase in % I form was shown 30 min after addition of insulin to muscles which had first been incubated for 210 min with glucose in absence of insulin (Table IV Exp B) In absence of glucose incubation with insulin for 210 min was also found to increase the % I form of the synthetase enzyme (Table IV Exp C) As previously described the lack of increase of % I form by insulin was seen after long time incubation with insulin in the presence of glucose and insulin (Fig 3 and Table IV

TABLE III Influence of short time incubation with insulin in absence and presence of glucose on glycogen synthetase activity of rat LA muscle

Synthetase enzyme activity after 5 min of incubation	Experimental conditions			
	No glucose (preincubation for 30 min)		Glucose 2 mg/ml (no preincubation)	
	Control	Insulin	Control	Insulin
% I form	16 9 ± 1 0	21 1 ± 0 8	19 6 ± 0 3	33 4 ± 2 3
I form mU/g wet tissue	160 ± 11	197 ± 5	161 ± 7	284 ± 48
(I+D) form mU/g wet tissue	950 ± 44	945 ± 45	8[illegible]3 ± 43	8[illegible]6 ± 106

Intact LA muscles were incubated for 5 min with and without insulin (10- IU/ml) in absence and presence of glucose 2 mg ml Muscles incubated without glucose were first preincubated for 30 min in absence of insulin and glucose to reduce the intercellular glucose concentration Two separate experiments are shown There are 4—6 observations in each group mean values ± S E are given I form and % I form are significantly increased by insulin at both experimental conditions (I+D) form was unaffected

TABLE IV Influence of long time incubation with insulin in absence and presence of glucose on the effect of insul n on glycogen synthetase activity and incorporat on of glucose C into glycogen in rat LA muscle

	Incubation time (min)	Experimental conditions					
		A With glucose insulin added at start		B With glucose insulin added at 210 min		C Without glucose insulin added at start	
		Control	Insulin	Control	Insulin	Control	Insulin
Synthetase I form	210	15 9±0 6	15 9±0 6	—	—	17 8±2 5	3? 1±3 1
	240	—	—	17 1±1 8	28 2±3 0	—	—
Glycogen mg/g wet tissue	210	3 80±0 14	8 05±0 55	2 88±0 09	—	? 70±0 04	2 55±0 28
	240	3 95±0 0?	8 30±0 70	2 43±0 12	3 21±0 24	2 30±0 25	2 84±0 53
Incorp of glucose C during the final 30 min CPM/mg wet tissue	240	64±8	273±?5	43±10	674±90	93±10	1100±160

Muscles were incubated for 210 and 240 min in absence or presence of insulin or glucose *In Exp B* no insulin was present for the first 210 min *In E p C* no glucose as present for the first ?10 min After 90 and 180 min of incubation muscles were transferred to fresh media After ?10 min of incubation some muscles were analysed for glycogen or synthetase I form the other muscles were incubated for an additional 30 min in presence of glucose 2 mg/ml For determination of the rate of incorporation of glucose C during these la t 30 min glucose C was added Three different experiments are shown Values are m ans of 4—5 observat ons ±S E Note the increase in I form by insulin in the experiment with low glycogen levels (Exp B and C)

TABLE V Influence of insulin on glucose 6 phosphate levels in rat LA muscle aft r var ous periods of incubation

		Incubation time (min)		
		30	120	240
Gluco e-6-P μ moles/ml ntra cellular fluid	Control	0 28±0 0?	0 ?3±0 02	0 16±0 01
	Insul n	0 3?±0 0	0 33±0 0?	0 35±0 04

Muscles were incubated n presence of glucose for the p riods ind cated There are 4—5 observa tions n each group Mean alues±S E are given

Exp A) It can be noted that glycogen levels were low in the first 2 expts showing an effect of insulin on the I form (Table IV Exp B and C) Glycogen levels were high in the experiment with no increase of I form (Table IV Exp A)

In the 3 expts just described the incorporation of glucose ^{14}C into glycogen was measured during the last 30 min of an incubation for 240 min In the expts with an increase of I form (Table IV Exp B and C) the effect of insulin on incorporation during this period was of the same high magnitude as that observed during the first 30 min of incubation (Table II) It was less pronounced in the absence of an in

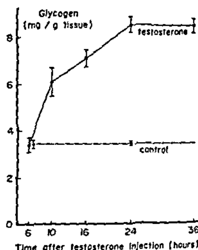


Fig 4 Influence of a single subcutaneous injection of testosterone propionate (100 mg/kg) on glycogen content in the LA muscle of prepubertal male rats Arachis oil was used as a vehicle for the hormone and was also injected into control rats Muscles were excised at the hours indicated and subsequently analysed for glycogen as described in Methods Data from two identical experiments were compiled Each point represents the mean of 8—10 rats S E × 2 is indicated by the length of the vertical bars

crease in I form (Table IV Exp A) Although less pronounced in this latter expt the effect of insulin on incorporation was quite clear (4 fold) It can be estimated that the CPM value (273 CPM/mg) corresponds to less than 0 2 mg glycogen/g tissue which explains the difficulty to demonstrate a significant increase in glycogen content during the final 30 min period

The effect of insulin on the level of glucose 6 P was measured in another set of experiments As shown in Table V glucose 6 P levels were not affected by the length of incubation in the presence of glucose and insulin In the absence of insulin there was a gradual decrease however The difference between the conditions was significant after 120 and 240 min of incubation

Time relationships for the influence of testosterone on glycogen synthesis and glycogen synthetase activity

The influence of testosterone was studied with testosterone propionate (Tp) in a *dose of 100 mg/kg b w This dose is well above that required for maximal effect* at least as far as membrane transport of amino acids are concerned (*cf* Arvill 1967 The glycogen content of the LA muscle was markedly increased by a single s c injection of Tp The increase was significant 10 but not 6 h after the injection (Fig 4 After 24 h the glycogen content had reached a maximum No further increase was observed after 36 h even when a second Tp injection was given 24 h after the first injection For comparison it could be mentioned that Tp did not influence the glycogen content of the diaphragm muscle as measured 24 h after injection of the hormone (control 2 07 ± 0 12 Tp 2 18 ± 0 13 mg/g diaphragm muscle 5 rats in each group

Although glycogen content was not increased 6 h after Tp injection an increase in incorporation of glucose ^{14}C into glycogen was observed when LA muscles were incubated for 2 h *in vitro* 6 h after injection of the hormone (Table VI The incorporation was increased also after 10 and 24 h

TABLE VI Time relationships for the influence of testosterone on glycogen synthetase activity and

	Hrs after injection of testosterone propionate		
	6 h		10 h
	Control	Testosterone	Control
Synthetase activity	19 4 ± 1 1	33 0 ± 1 5	20 2 ± 1 2
I form			
I form mU/g wet tissue	156 ± 17	253 ± 21	154 ± 10
(I+D) form mU/g wet tissue	805 ± 30	81? ± 46	765 ± 38
Incorp of glucose C *in vitro*			
CPM/mg wet tissue	140 ± 30	5580 ± 390	90 ± 16
Glycogen mg/g wet tissue	3 53 ± 0 53	3 4? ± 0 18	3 83 ± 0 37

For legend see next page

The activity of the synthetase enzyme was found to be increased by injection of Tp An increase in the I form of the enzyme was observed after 6 10 and 16 h but not after 24 h (Table VI) In a separate experiment no influence of Tp was found 4 h after its injection the % I form was 17 7 ± 1 6 and 18 3 ± 2 0 for control and Tp groups respectively (5 rats in each group) At all periods investigated no increase in total enzyme activity was observed When the values from 6—24 h were added to give a higher number of observations there was still no difference between control and Tp groups

The influence of testosterone on the level of glucose 6 P was measured 10 and 24 h after the injection of Tp At both periods there was an increase 10 h 0 54 ± 0 05 *vs* 0 81 ± 0 07 24 h 0 52 ± 0 09 *vs* 0 94 ± 0 07 Values are μmol/ml intracellular fluid there were 5—6 observations in each group

Discussion

In characterizing the influence of a hormone it is important to elucidate its effects in relation to time Previous studies of the *in vitro* effects of insulin on the glycogen synthetase enzyme in muscle have been performed on isolated rat diaphragms incubated with the hormone for no longer than 30 min (Villar Palasi and Larner 1961 Craig and Larner 1964 Danforth 1965 Sovik 1966) In the present study the influence of insulin on glycogen synthesis and synthetase activity was investigated *in vitro* for various periods from 5 to 240 min using LA muscle preparations from prepubertal male rats Temporal relationships in the stimulation of glycogen synthesis were also studied *in vivo* with testosterone For both hormones the length of stimulation was of importance for the activation of the synthetase enzyme The time scale differed however For testosterone there was a lag period of 4—6 h before any increase of synthetase activity was obtained For insulin an increase of the active I form of the synthetase enzyme was found after only 5 min of incubation The lag period for the effect of testosterone on glycogen synthesis is in good agreement with

rate of incorporation of glucose ¹⁴C into glycogen

10 h	16 h		24 h	
Testosterone	Control	Testosterone	Control	Testosterone
36 3±2 0	20 9±1 7	29 9±2 0	18 2±1 9	16 9±1 3
297±25	163±27	268±17	142±14	147±9
820±58	791±39	845±18	877±79	859±60
1710±163			120±27	1190±140
5 52±0 19	3 38±0 21	6 71±0 37	3 66±0 30	7 84±0 58

See legend to Fig 4 Synthetase enzyme activity and glycogen content were measured at the hrs indicated The rate of incorporation of glucose ¹⁴C into glycogen was determined *in vitro* This was done 6 10 and 24 h after Tp injection LA muscle preparations were incubated for 2 h as described in Methods There are 4—5 rats in each group Mean values ± S E are given No statistical difference was found for synthetase (I+D) form when control and Tp groups were compared

that shown for its influence on membrane transport of amino acids and glucose in the LA muscle Arvill (1967) demonstrated an increased transport of the model amino acid α aminoisobutyric acid (AIB) 6 h after injection of testosterone and Bergamini and Pagni (1969) could show a lag period of 6 5 h for membrane transport of xylose The effects of testosterone on membrane transport and glycogen synthesis therefore appear to occur at the same time The reason for the delayed action of the hormone is still unknown The lag period is also observed with intravenous injection of testosterone (Arvill 1967)

The rapid effect of insulin on the synthetase enzyme was independent of the addition of glucose to the incubation medium This independency has also been shown in the diaphragm muscle (Villar Palasi and Larner 1961) Interestingly a graded response to insulin of the % I form of the synthetase enzyme was found in the LA muscle when the insulin concentration was increased from 10⁻³ to 10 IU/ml The increase of % I form was time dependent It was continuously reduced during incubation in the presence of glucose and no longer present after 150 min Glycogen content increased linearly for 150 min however Thus the level of I form does not seem to be the only indicator of the activity of the rate limiting synthetase enzyme It is possible that insulin during long time stimulation increases the activity of the synthetase enzyme by other mechanisms than an increase of I form This possibility will be discussed at the end of this section During testosterone stimulation the % I form was steadily elevated throughout the period of increased accumulation of glycogen After that period it was reduced to control level For both insulin and testosterone the incorporation of glucose ¹⁴C into glycogen was increased after long time stimulation when there was no increase in I form This also suggests the existence of other mechanisms operating to activate the synthetase enzyme

In both the experiments with insulin and with testosterone glycogen was accumu

lated up to a level of 8 5—9 0 mg/g wet tissue This level is similar to that found in the rat diaphragm after insulin stimulation *in vivo* (Sovik 1966 Adolfsson 1972) and probably represents the maximal glycogen content obtainable in rat muscle tissue During long time stimulation by insulin and testosterone the % I form of the synthetase enzyme was reduced to control level when this glycogen concentration was reached In some experiments there was even a decrease When long time stimulation was not associated with an increase in glycogen content as in the experiments with incubation in absence of glucose the increase in I form by insulin persisted An inverse relationship between % I form and glycogen content has been demonstrated in rat diaphragm muscle by Danforth (1965) Although glycogen and synthetase activity were not measured on the same muscle in the present study such an inverse relationship is suggested by the results obtained from muscles incubated for various periods with insulin

The mechanism for the decrease in I form by glycogen has been elucidated by Larner *et al* (1968) Glycogen was found to decrease the activity of the phosphatase enzyme that converts D form (phosphorylated) to I form (dephosphorylated) There are no reports showing an influence of insulin on this interconverting enzyme in muscle tissue According to Shen *et al* (1970) there is an influence of the hormone on the other interconverting enzyme in the synthetase enzyme system. This enzyme is a protein kinase It converts I form to (inactive) D form and is partly dependent on cyclic AMP In the study by Shen *et al* insulin was found to increase the dependence on cyclic AMP thereby decreasing I to D form conversion at unchanged cyclic AMP levels A decrease in cylic AMP would also slow down the inactivation of the I form However such a decrease has not been convincingly shown for insulin in muscle tissue It is not known how testosterone acts on the two interconverting enzymes in the synthetase enzyme system

Apart from influencing the relation between the two activity forms of the enzyme I form and D form an increase in synthetase activity by an increase in the amount of enzyme protein during hormonal stimulation may be considered Such an influence is more probable during sustained stimulation *e g* with repeated injections of Tp than during the acute effects of insulin In the present study no increase of (I+D) form activity was found for either hormone

The increase in glycogen content observed in the present study may not only be the result of *an enhancement of glycogen synthesis but also of a decrease in glycogen* degradation However this possibility is not supported by reports in the literature The active a form of the glycogen degradating phosphorylase enzyme has been found to be unaffected by testosterone in LA muscles of castrated rats (Turner and Leonard 1969) Furthermore testosterone treatment increased total phosphorylase activity instead of decreasing it (Turner and Leonard 1969 Bergamini *et al* 1969) Concerning the influence of insulin on phosphorylase activity studies on isolated rat diaphragms have shown no decrease (Craig and Larner 1964 Torres *et al* 1968) with the exception that insulin reduces the increase of a form induced by epinephrine (Torres *et al* 1968)

It was mentioned previously in the discussion that an activation of the synthetase enzyme by other mechanisms than an increase in its I form is likely during hormonal stimulation. It is known that synthetase activity is influenced by glucose 6 P and adenine nucleotides as well as other metabolites in concentrations likely to be present in muscle tissue (Piras *et al* 1968, Piras and Staneloni 1969). Both forms of the enzyme are inhibited by ATP, ADP and inorganic phosphate. Glucose 6 P reverses the inhibition of the I form but unless very high concentrations of glucose 6 P are reached (10 mM) there is only a minor reversion of the inhibition of the D form. As tissue levels of ATP, ADP and inorganic phosphate are quite stable in muscle under aerobic conditions (Piras and Staneloni 1969 and preliminary results from this laboratory), variations in the concentrations of glucose 6 P will be of importance for synthetase activity. In the present study there was no significant increase in glucose 6 P with length of incubation but an increase compared to controls was observed after longer periods of incubation. During testosterone stimulation glucose 6 P was increased both 10 and 24 h after injection of the hormone. These data are compatible with an activation by glucose 6 P of the synthetase enzyme in the LA muscle during hormonal stimulation. A similar activation of the enzyme is probable in the rat diaphragm muscle after an intraperitoneal injection of insulin (Sovik 1966). It was also suggested in a study of the *in vitro* effect of insulin on the perfused rat heart (Adolfsson *et al* 1972). However further investigations will be needed to elucidate the different mechanisms involved in the regulation of muscle glycogen synthesis during hormonal stimulation. The LA muscle offers special advantages for such investigations as it is sensitive to two hormones stimulating glycogen synthesis, i e insulin and testosterone. Studies are in progress to elucidate the combined influence of these two hormones on glycogen synthetase activity.

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From the Institute of Medical Biochemistry University of Oslo Norway

The Effect of Insulin on Epinephrine and Glucagon Inactivated Glycogen Synthetase I in the Isolated Perfused Rat Liver

By

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Abstract

HOSTMARK A T *The effect of insulin on epinephrine and glucagon inactivated glycogen synthetase I in the isolated perfused rat liver* Acta physiol scand 1973 88 248—255

The effect of insulin on the reactivation of the epinephrine or glucagon inactivated glycogen synthetase I in the isolated perfused rat liver has been investigated. Livers from fed rats and fasted rats were perfused with Krebs Ringer bicarbonate buffer using either a recirculating or non recirculating apparatus. Inactivation of the glycogen synthetase I was observed following the administration of epinephrine or glucagon. It was demonstrated that insulin added 10—15 min after epinephrine or glucagon promoted the reactivation of synthetase I without altering the perfusate glucose concentration. Reactivation by insulin was also observed when livers were perfused with a glucosefree medium in a non recirculating system. It is concluded that this action of insulin is not dependent upon the presence of glucose

Following the administration of insulin to alloxan diabetic rats there is an increase in liver glycogen and in glycogen synthetase activity (Steiner 1964). In the 18 h fasted dog liver glycogen synthetase I activity rapidly increases upon the injection of insulin (Bishop and Larner 1967). Furthermore Gold (1970) found that insulin restored the synthetase activation in the liver of the alloxan diabetic dog. Insulin activation of synthetase has also been reported by Bishop and his co workers (1971).

On the other hand some investigators have not been able to demonstrate any effect of insulin on the liver glycogen synthetase in the isolated perfused rat liver (Glinsmann Pauk and Hern 1970). After the demonstration by De Wulf and Hers in 1967 that glucose itself could activate synthetase the concept that the insulin effect might be explained by alterations of intracellular glucose concentration had to be considered. Glucose activation of synthetase has recently been demonstrated in the perfused liver by Buschiazzo Exton and Park (1969) and by Glinsmann *et al* (1970)

Key words Insulin epinephrine glucagon glycogen synthetase perfused liver

In the present study the effect of insulin on the reactivation of the epinephrine and glucagon inactivated glycogen synthetase has been investigated in the isolated perfused rat liver The results indicate that insulin *per se* promotes the reactivation of the synthetase

Experimental Procedure

The liver perfusion technique
Two types of perfusions were performed In the first type the recirculating apparatus de cribed by Seglen and Jervell (1969) was used. The perfusions were carried out at 30 C to reduce the oxygen requirement and to prevent loss of potassium (Frimmer and Kreh 1971 A non recirculating perfusion system was made by a modification of the Seglen and Jervell apparatus

Perfusate
The perfusion medium (total volume 30 ml) consisted of Krebs Ringer bicarbonate buffer with added bovine albumin (0 9 %) heparin (0 25 ml) and human erythrocytes (5 ml giving 16 % packed cell volume) The erythrocytes were 3—5 weeks old and were washed twice in 3 olumes oxygenated 0 9 % NaCl solution before use (Hems *et al* 1966) In order to a oid the vaso-constrictor effect of epinephrine the alpha blocking agent phentolamine (Regitin) as added to the perfusion medium in some experiments During perfusion the flow rates of bile and of perfusate vere relativly constant (aproximatly 0 4 μl/min/g and 1 5—2 0 ml/min/g respectively)

Sampling
Male Wistar rats weighing 130—180 g were used as liver donors Hepatectomy was performed under Nembutal anesthesia (30 mg Nembutal per kg b wt) by the method of Seglen and Jervell (1969) Tissue samples were obtained as described by these authors with the exception that the tissue samples were smaller (100—200 mg) Only one sample wa taken from each liver lobe since ligation may influence the adjacent tissue The sampling sequence was random The samples were frozen in liquid nitrogen and usually assayed on the day of perfusion The parameters used for judgment of the vitality of the organ were the flow rates of bile and perfusate and the gross appearance of the organ

Glycogen synthetase activity
Glycogen synthetase activity was assayed by the method of Thomas Schlender and Larner (1968) in a $8000 \times g$ supernatant from 1 10 (w/v) homogenate Radioactivity as determined in a liquid scintillation counter Packard Model 3003 Homogenization was carried out with a Potter Elvehjem homogenizer The homogenization and enzyme assay were performed in a buffer contain ng Tris HCl 50 mM KF 100 mM EDTA 5 mM pH 8 2 This pH was found to be optimal w th the buffer system used Enzyme act vity was calculated as nanomoles glucose transferred from UDPG to glycogen per minute per mg protein The results are presented as per cent of the zerotime value i e the value at the end of preperfusion or equilibrium period

Prot in Analysis
Protein was determined in 50 μl of the $8000 \times g$ supernatant by the b uret method of Zamenhof (1957) The standard curve for bovine serum albumin in the buffer used was l near over the experimental range

Gluc se Analysis
The glucose concentration in the p rfusate was determined in 100 μl samples by the glucose oxidase method

Materials

Epinephrine (Sigma Lot E 101 B-087) was prepared as a stock solution containing 1 mg ep nephrine/ml 5 mM HCl This solution was freshly prepared every day A few minutes before use it was diluted 1 100 with the perfusion medium and 250 μl was then aded to the 30 ml perfusate The final concentrat on was thus 0 8 μg per ml

Glucagon (a gift from Eli Lilly Research Laboratories Indianapolis Lot 258 1011 B 1) was d ssolved in 5 mM HCl on the day of perfusion giving a stock solution of 1 mg/ml As with epinephrine the stock solution was diluted with the perfu ion medium a short time before use The final concentration was 1 or 10 μg/ml

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The Effect of Insulin on Epinephrine and Glucagon Inactivated Glycogen Synthetase I in the Isolated Perfused Rat Liver

By

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Abstract

HOSTMARK A T *The effect of insulin on epinephrine and glucagon inactivated glycogen synthetase I in the isolated perfused rat liver* Acta physiol scand 1973 *88* 248—255

The effect of insulin on the reactivation of the epinephrine or glucagon inactivated glycogen synthetase I in the isolated perfused rat liver has been investigated Livers from fed rats and fasted rats were perfused with Krebs Ringer bicarbonate buffer using either a recirculating or non recirculating apparatus Inactivation of the glycogen synthetase I was observed following th administration of epinehprine or glucagon It was demonstrated that insulin added 10—15 min after epinephrine or glucagon promoted the reactivation of synthetase I without altering the perfusate glucose concentration Reactivation by insulin was also observed when livers were perfused with a glucosefree medium in a non recirculating system It is concluded that this action of insulin is not dependent upon the pre ence of glucose

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Key words Insulin epinephrine glucagon glycogen synthetase perfused liver

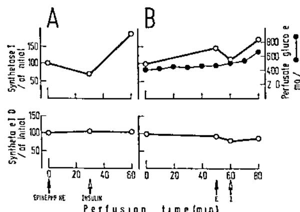


Fig 2 Reactivation of glycogen synthetase I by insulin in the liver from fed rats Livers from male fed rats were perfused in a recirculating system Zero time samples were determined after a 30 min equilibrium perfusion At the times indicated epinephrine (giving 0 8 μg/ml final concentration 2 U/ml) were added in single doses Regitin (3 μg/ml) was added at the same time as epinephrine Three different experiments have been performed with essentially the same results *A* represents the mean values of 2 expts where glucose was added to the medium at a concentration of 100 mg % and insulin added 30 min after administration of epinephrine *B* represents the result of a single perfusion under slightly different conditions Glucose was added at a concentration of 150 mg % and insulin added 10 min after administration of epinephrine 100 % I activity was 1 63 mmol/min × mg protein (*A*) and 2 06 nm/min × mg protein (*B*) The corresponding values for (I+D) activity were 9 43 and 7 65 nmol/min × mg protein

effect of a single dose of epinephrine on the liver synthetase I levels was maintained for at least 60 min

The effect of insulin on this system is shown in Fig 2 When insulin was added 30 min (*A*) or 10 min (*B*) after administration of epinephrine there was a reactivation of the synthetase The glucose concentration curve in B shows that epinephrine has the same effect as in the experiments in Fig 1

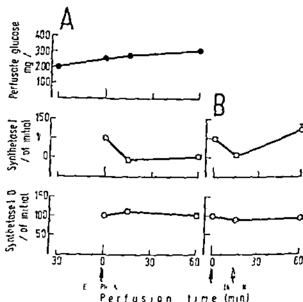
The reactivation of glycogen synthetase I by insulin in the epinephrine treated liver from animals fasted for 48 hours

In order to study the effect of insulin with a minimum change in perfusate glucose concentration animals were starved 48 hours prior to sacrifice and 200 mg % glucose was added to the perfusion medium Again an inactivation of synthetase I was noted upon the administration of epinephrine (Fig 3) The epinephrine effect on the enzyme was marked and maintained for at least 60 min as can be seen from Fig 3 *A* However when insulin was added to the perfusion medium 15 min after epinephrine the synthetase I was fully reactivated 45 min after addition of insulin (Fig 3 *B*) The total (I+D) activity remained unaltered during the perfusion There was a relatively small and constant increase in perfusate glucose concentration from the onset of the preperfusion (Fig 3 *A* top) In these experiments glucose concentration in the perfu ion medium did not appear to be influenced by the addition of epinephrine

Perfusions using a non recirculating medium

The non recirculating system was used to investigate the action of insulin on the glycogen synthetase system in the absence of glucose in the medium It was observed that livers from rats starved for 2—3 days and perfused with a non recirculating

Fig 3 Reactivation by insulin of the glycogen synthetase I in the liver from 48 h starved rats The livers from male rats starved for 48 h were perfused with a recirculating medium (Krebs Ringer bicarbonate buffer containing 200 mg % glucose bovine albumin—3 %—and washed human red cells 16 % PCV) After a 30 min equilibrium period the first liver samples were taken Epinephrine and insulin were added in single doses at the times indicated giving final perfusate concentrations of 0 8 μg/ml and 2 U/ml respectively Regitin (3 μg/ml) was added 15 min after epinephrine in both A and B The mean values ± S E of 3 (A) or 4 (B) perfusions are indicated Φ Zero time glycogen synthetase I activity (= 100 %) was 3 90 nmol/min × mg protein in A and 1 65 nmol/min × mg protein in B The corresponding (I + D activities were 11 95 and 7 13 respectively



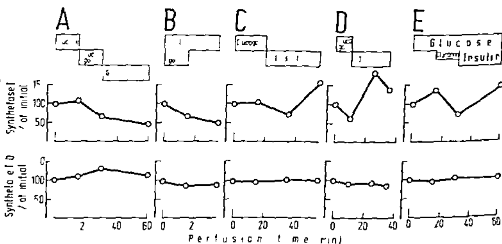


Fig 4 The effect of glucose glucagon and insulin on the fasted liver perfused without recirculation Gl cogen synthetase response was investigated in livers from male rats fasted for 24 h (A, B C E) or 48 h (D) The livers were perfused (without recirculation) with buffers (Krebs-Ringer bicarbonate red cells-albumin) which during the time intervals indicated contained glucose (100 mg final concentration) glucagon (1 μg/ml A B and E 10 μg C and D) or insulin (0 08 U/ml C and D 0 5 U/ml E) The preperfusion time was 5—15 min 100 % I activities in nmol/min × mg protein 1 96 (A) 2 80 (B) 0 82 (C) 0 67 (D) and 2 26 (E) The corresponding (I + D) activities 8 75 (A) 11 80 (B) 10 23 (C) 8 92 (D) and 10 27 (E)

glucose free medium soon became discoloured and exhibited a reduced flow rate of both perfusate and bile These difficulties were overcome by using livers from rats

starved for only 24 h (in all but 1 expt) Glucagon instead of epinephrine was used for prestimulation since the actions of this hormone also are mediated by activation of adenyl cyclase Glucagon is however of great physiological importance for the liver Because of the large volumes of perfusion medium necessary in these experiments the preperfusion period was shortened or omitted

A series of experiments have been performed under slightly differin conditions Some typical experiments are shown in Fig 4 It can be seen (Fig 4 A E) that glucose activates glycogen synthetase under these conditions Furthermore in the presence (Fig 4 E) and in the absence of glucose (Fig 4 C D) the enzyme after inactivation by glucagon was reactivated by insulin It has further been observed that the glucagon inactivated enzyme is not reactivated by glucose if insulin is absent (Fig 4 A B)

Discussion

The results obtained in the present work are based upon a total of 30 perfusion expts *of the isolated rat liver under somewhat different conditions* The most important finding is an activation of glycogen synthetase by insulin under a series of different experimental conditions Furthermore this work has confirmed some results obtained by previous workers in this field This concerns inactivation of glycogen synthetase by glucagon and epinephrine and activation of the enzyme by glucose

The problem of whether insulin has a direct effect on liver glycogen synthetase has been a subject of much controversy It has been stated that all efforts to demonstrate an activation by insulin of mouse liver synthetase have given negative results (Hers *et al* 1970) These workers point out that it is doubtfull that the large increase in glycogen synthetase which is induced by a glucose load can be explained by pancreatic effects Hers has furthermore suggested that the glucose effect is due to glucose stimulation of phosphorylase phosphatase which promotes dephosphorylation of phosphoryla e *a* This latter enzyme is an inhibitor of the synthetase phosphatase Thus the glucose effect on glycogen synthetase could be due to alterations in the level of phosphorylase *a* Glinsmann *et al* (1970) have demonstrated that in the perfused rat liver hyperglycemia causes a rapid inactivation of phosphorylase *a* and a conversion of glycogen synthetase from a G6P dependent to G6P independent form This effect is not altered by the presence of added insulin These authors could not demonstrate any action of insulin on hepatic glucose uptake or glycogen synthesis in the isolated liver

On the other hand it appears that insulin is in some way necessary for activation of synthetase by glucose since both the alloxandiabetic rat (Steiner 1966) and the pancreatectomized dog (Bishop *et al* 1971) have a low I synthetase activity even though the blood glucose concentration is very high Kreutner and Goldberg (1967) suggested that the glucose glucocorticoid activation of rat liver synthetase was mediated by insulin and Gold (1970) found that the time dependent activation of liver synthetase which was lost in alloxan diabetes could be restored by insulin

Villar Palasi *et al* (1970) demonstrated a marked and rapid insulin activation of synthetase in normal dog liver Blatt and Kim (1971) have demonstrated that insulin causes activation of glycogen synthetase in tadpole livers Recent studies by Mondon and Burton (1971) and by Ottolenghi *et al* (1971) indicate that the parasympatetic nerve system may be involved in the insulin action

The results obtained in the present work demonstrate that insulin can promote reactivation of the epinephrine inactivated synthetase when the glucose concentration in the perfusion medium is relatively constant This insulin effect has been demonstrated in the recirculating perfused liver both from fed and fasted animals as well as during perfusion of the liver in a non recirculating system The results obtained from non recirculating perfusions of livers from fasted rats suggest that insulin alone can reactivate synthetase I

The present study gives no indication of the mechanism behind the synthetase reactivating action of insulin In the various experiments reported here the total (I+D) activity remained unchanged This finding rules out the possibility that the insulin effect proceeds via direct inhibition or stimulation of the synthetase I or D enzymes For the same reason and also because of the relatively short experimental periods synthesis of new enzyme protein can also be ruled out Thus insulin must in som way effect the interconverting enzymes *i e* the kinase (I→D) or phosphatase (D→I) It is known that insulin causes a decrease in liver cyclic AMP concentration (Exton and Park 1968) This might account for the augmentation in per cent of the glycogen synthetase in the I form By stimulation of protein kinase cyclic AMP leads to an increase in phosphorylase *a* which according to Hers *et al* (1971) inhibits synthetase phosphatase The effect on glycogen synthetase can be at least partially explained by hormonal action on cyclic AMP levels As a result of the decreased cyclic nucleotide concentration due to insulin one would expect a decreased stimulation of the synthetase kinase and a release from the inhibition of synthetase phosphatase by phosphorylase *a* In light of the important discovery by Hers *et al* (1971) that phosphorylase *a* inhibits synthetase phosphatase it would be of interest to compare liver phosphorylase activities with those of synthetase during the perfusion However Glinsmann and Mortimore (1968) observed that the inhibition by insulin of glucagon induced glycogenolysis in perfused livers was not associated with diminished phosphorylase activation

Another possible mechanism for the action of insulin may be through its influence on ion transport as discussed by Horn *et al* (1972) Their investigation of the effect of insulin on muscle synthetase has shown that alterations in the ionic milieu can modify the insulin response of the synthetase

Glycogen is known to inhibit glycogen synthetase phosphatase D (Larner *et al* 1968 De Wulf *et al* 1970) The mechanism of the insulin effect therefore might be due to an increase in glycogen level In the present study reactivation by insulin of synthetase was found in the liver from both fed and fasted rats It therefore seems unlikely that alterations in liver glycogen levels are responsible for the insulin effect reported here

Whatever the mechanism may be, the data from the present study strongly suggest that insulin *per se* does have an activating effect on the glycogen synthetase I in the liver

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Acta physiol scand 1973 88 256—266

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Effects of Severe Systemic Hypoxia on Myocardial Energy Metabolism

By

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Abstract

Fellenius E and R Samuelsson *Effects of severe systemic hypoxia on myocardial energy metabolism* Acta physiol scand 1973 88 256—266

By the use of freeze clamp technique simultaneous tissue samples were taken from the right atrial and the right ventricular muscle of the working heart in anesthetized dogs during a hypoxia recovery cycle consisting of 4 min of severe systemic hypoxia followed by a period of recovery of equal duration In order to study the energy metabolism of the two parts of the heart the tissue samples were analyzed with respect to their content of creatinephosphate adenine nucleotides pyruvate lactate dihydroxyacetonephosphate α glycerophosphate and malate During aerobic controlled ventilation the ventricular tissue was found to contain higher concentrations of high energy phosphate compounds than the atrial tissue During the period of hypoxia the atrial and ventricular tissue contents of creatinephosphate were reduced to about 10 % whereas ATP was reduced merely to about 70 % of the control values The rate of the anaerobic glycolysis was found to proceed more rapidly in the ventricular muscle thereby indicating a higher rate of energy metabolism than in the atrial muscle

An inadequate oxygen supply to the heart brings about a rapid deterioration of the transmembrane electrical characteristics of the heart muscle cells (Trautwein Gottstein and Dudel 1954 Webb and Hollander 1956 Kardesch Hogancamp and Bing 1958) Furthermore an inadequate oxygen supply induces a rapid shift of the myocardial metabolism from efficient aerobic to less efficient anaerobic pathways of energy production (cf Bing 1965 Katz 1968 Newsholme 1970) which results in a rapid decrease of the myocardial tissue concentrations of the high energy phosphate compounds adenine triphosphate (ATP) and creatinephosphate (CP) (Feinstein 1962 Krause and Wollenberger 1965 Braasch *et al* 1968)

Among the alterations of the characteristics of the myocardial transmembrane electrical activity which may be recorded as a consequence of an insufficient oxygen supply, shortening of the duration of the action potential constitutes the earliest and most striking change Available evidence indicates that ATP is the basic energy source which is necessary for the maintenance of normal transmembrane electrical

activity of heart muscle cells (de Mello 1959, Keynes 1965 Dydynska and Harris 1966) whereas CP serves as a store of energy rich phosphate for rephosphorylation of adenosinediphosphate (ADP) according to the Lohmann reaction (Lohmann 1934)

Using an intracardiac suction electrode technique Samuelsson (1973) made simultaneous recordings of monophasic action potentials (MAP) from the right atrium and the right ventricle in the parasympathetically and sympathetically denervated dog heart during a sequence of 4 min of severe systemic hypoxia followed by a period of recovery of equal duration In these experiments the monophasic action potential duration (MAPD) which is a reliable approximation of the transmembrane action potential duration (Hoffman et al 1959 Churney and Ohshima 1964) was abbreviated earlier during the hypoxic phase and normalized earlier during the recovery phase in the atrial than in the ventricular recording This temporal disparity with regard to the alteration of the MAPD between the atrial and ventricular recordings may reflect a difference with respect to the rate of utilization and/or the magnitude of supply of high-energy phosphate compounds between the two different parts of the heart

The present work was undertaken in order to study this question and to examine the temporal relationship between the abbreviation of the MAPD (cf Samuelsson 1973) and the decrease in the myocardial concentration of CP and ATP in the heart in situ exposed to severe systemic hypoxia

Methods and experimental procedure

The experiments were performed on mongrel dogs of both sexes [illegible] 19 kg After the induction of anesthesia (Nembutal® 25 mg/kg) [illegible] artificially respirated with high frequency positive pressure ventilation [illegible] 1971)

The thorax was opened by splitting the sternum and the pericardium [illegible] right atrial appendage and the right ventricular wall lay free [illegible] parasympathetically by vagal division in combination with i v adm[illegible] sulphate (2 mg/kg) and sympathetically by destruction of the stellate [illegible] with i v administration of propranolol (Inderal® 1 mg/kg)* Serial [illegible] blood samples was accomplished from a catheter introduced int[illegible] respiration was regulated so that the arterial P_{O_2} was greater [illegible] acid base status was kept within the following limits pH 7 40±0 05 [illegible]

For measurement of the arterial pH and P_{O_2} an Acid Bas[illegible] connected to a Blood Micro System Type BMS 2 b and a [illegible] (Radiometer Copenhagen Denmark) were used The P_{CO_2} was d[illegible] Siggaard Andersen Curve Nomogram for human blood using the A[illegible] Andersen 1967) The concentrations of potassium and sodium in th[illegible] were determined with an Eppendorf flame photometer (Nethler and H[illegible] Germany)

Severe systemic hypoxia was induced by exchanging the air in th[illegible] 100 % nitrogen During the recovery phase the animal was again [illegible] pacemaker catheter (USCI No 5652) localized in the upper part of the [illegible] used to keep the heart rate constant throughout the experiment

Sampling procedures

The tissue sampling was performed simultaneously from the right [illegible] right ventricular wall by means of the freeze clamp technique [illegible]

* Inderal was kindly supplied by Scanmeda AB Gothenburg [illegible]

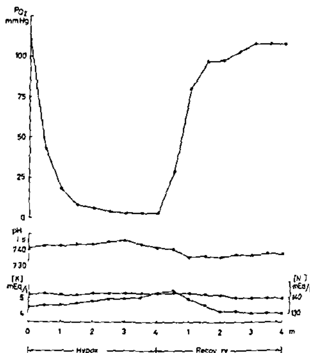


Fig. 1. Arterial P_{O_2} and pH and arterial plasma concentrations of sodium and potassium measured every 30th second in 1 of the 4 dogs exposed to 4 min of severe systemic hypoxia followed by a period of recovery of equal duration.

Pistau and Schoffa (1960). The frozen tissue was rapidly cut free from the heart and plunged into liquid nitrogen, where it was kept until the analytical procedures were started. Because of the destruction of the heart resulting from the sampling technique, the samplings were performed only once in each animal. The samples were taken after periods of 1, 2.5 and 4 min of severe systemic hypoxia and after 2 and 4 min of recovery following a period of 4 min of severe systemic hypoxia. Each of these experimental periods comprised 2 dogs. Control experiments, in which no hypoxia was induced, were performed in 6 animals.

In 4 dogs arterial blood sampling was performed every 30th second during a cycle of 4 min of hypoxia followed by a period of recovery of equal duration. The blood samples were collected in plastic syringes (Mediplast AB, Gothenburg, Sweden) and kept in ice water pending the analysis (Hilty and Karendal 1969), which was done immediately after the experiment. Samples intended for the electrolyte analysis were centrifuged after the experiment and the plasma was separated from the blood cells.

Chemical analyses

About 1 g of the frozen tissue was ground in a percussion mortar containing liquid nitrogen and dissolved in 3 ml of chilled 6 % $HClO_4$ (Lowry *et al.* 1964). After homogenization, centrifugation and removal of the $HClO_4$ with KOH, the concentrations of the different metabolites were determined by enzymatic methods using a Zeiss spectrophotometer model PMQ II (Carl Zeiss, Wurttemberg, West Germany). The concentrations of lactate, malate, α-glycerophosphate (α-GP), pyruvate and dihydroxyacetonephosphate (DAP) were determined by the method of Hohorst *et al.* (1959), ATP by the method of Lamprecht and Trautschold (1962), ADP and adenosine monophosphate (AMP) by the method of Adam (1962) and CP by the method of Lamprecht and Stein (1962).

Statistical analyses

Student's t test was used for statistical analyses of the differences in concentrations between the atrial and the ventricular samples taken from the same heart for the respective metabolites.

Results

The arterial blood P_{O_2} and pH, arterial plasma concentrations of sodium and potassium during the hypoxia recovery cycle

During the hypoxic period the arterial P_{O_2} decreased rapidly, usually reaching a

TABLE I Control values of right atrial and right ventricular concentrations of pyruvate lactate DAP α GP and malate and calculated ratios of lactate/pyruvate and α GP/DAP concentrations Mean values ± S D

	Number of hearts	Pyruvate	Lactate	DAP	α GP	Malate	Lactate / Pyruvate	α GP / DAP
		μmol/g wet weight						
Right atrial appendage	6	0.104 ± 0.017	1.38 ± 0.24	0.032 ± 0.008	0.16 ± 0.01	0.101 ± 0.008	13.5 ± 1.3	6.0 ± 1.0
Right ventricular wall	6	0.070 ± 0.010	1.16 ± 0.17	0.038 ± 0.006	0.22 ± 0.07	0.123 ± 0.015	16.6 ± 0.6	6.0 ± 0.8
Statistical signif of the diff between atrial and ventricular values $p < 0.05$		—	—	—	—	—	—	—

value below 25 mm Hg during the first minute (*cf* Fig 1) Thereafter the decrease proceeded at a slower rate until it gradually approached a value below 5 mm Hg During the recovery period a value above 75 mm Hg was reached as a rule in the first minute whereafter the increase proceeded at a slower rate finally reaching a value approximately equal to the initial value Alterations in the arterial pH recorded during the experimental period appeared as a rule in association with the change of the ventilation gases These changes were relatively moderate and no divergence from the initial value exceeding 0.10 pH units was recorded (*cf* Fig 1)

The arterial plasma concentration of sodium remained largely constant throughout the experimental period in all cases Irregularly appearing alterations when recorded were very small and not correlated to alterations of the aerobic state of the blood The arterial plasma concentration of potassium however was in all cases moderately increased during the period of hypoxia and decreased again to approximately the initial value during the period of recovery The increases in the concentrations of potassium recorded were in the range of 0.5—1.5 mEq/l (*cf* Fig 1)

Effects of hypoxia and recovery from hypoxia on the concentrations of certain anaerobic metabolites in the right atrial and the right ventricular muscle

The tissue concentrations of pyruvate lactate DAP α GP and malate found in the control group together with the calculated ratios of lactate/pyruvate and α GP/DAP are presented in Table I It can be seen in the table that the difference in concentration between the atrial and ventricular muscle was not significant for any of the metabolites or for the calculated ratios

As can be seen from Fig 2 the concentrations of lactate α GP and malate the 3 hydrated end products of the anaerobic metabolism were increased in the atrial as well as in the ventricular muscle during the period of hypoxia Quantitatively the

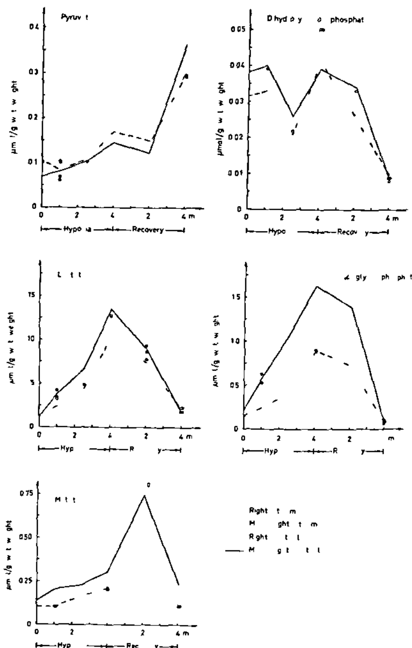


Fig 2 Right atrial and right ventricular tissue concentrations of pyruvate lactate DAP α GP and malate measured during a hypoxia recovery cycle consisting of 4 min of severe systemic hypoxia followed by a period of recovery of equal duration

accumulation of lactate far exceeding that of α GP and malate demonstrating that pyruvate constitutes the main proton acceptor of the heart muscle during anaerobic metabolism Malate in contrast to lactate and α GP continued to accumulate

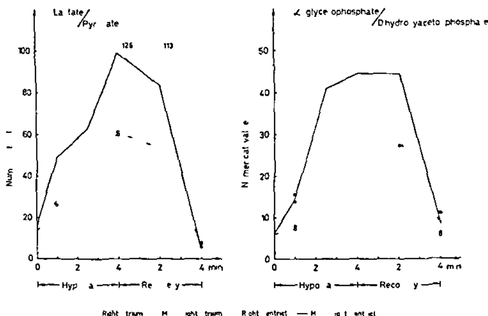


Fig 3 Lactate/pyruvate and α GP/DAP concentration ratios measured in the right atrial and the right ventricular tissues during a hypoxia recovery cycle consisting of 4 min of severe systemic hypoxia followed by a period of recovery of equal duration

during the early part of the recovery period The concentrations of all of these hydrated metabolites decreased to the control levels during the recovery period The tissue contents of pyruvate and DAP fluctuated irregularly throughout the experimental period The fluctuations did not seem to be correlated to the alterations of the oxygen supply

TABLE II Control values of right atrial and right ventricular concentrations of CP and adenine nucleotides The values are expressed mean ± S D

	Number of hearts	CP	ATP	ADP	AMP	ATP ADP AMP
		μmol/g wet weight				
Right atrial appendage	6	4 54 ± 0 27	3 33 ± 0 13	0 564 ± 0 012	0 063 ± 0 006	3 95 ± 0 13
Right ventricular wall	6	8 79 ± 0 53	5 13 ± 0 24	0 838 ± 0 030	0 067 ± 0 005	6 03 ± 0 27
Statistical significance of the diff between atrial and ventricular values $p < 0.05$		+	+	+	−	+

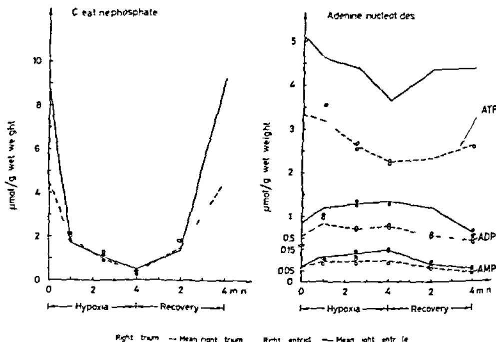


Fig. 4. Right atrial and right ventricular tissue concentrations of CP and adenine nucleotides during a hypoxia recovery cycle consisting of 4 min of severe systemic hypoxia followed by a period of recovery of equal duration.

Both the lactate/pyruvate and α GP/DAP ratios, reflecting the state of the anaerobic glycolysis of the tissues (*cf.* Bing 1965), were markedly increased during the period of hypoxia and decreased again to the initial levels during the recovery period in a clear-cut way (see Fig. 3).

The kinetics of the changes in concentration of pyruvate and DAP showed no essential divergence between the atrial and the ventricular muscles throughout the experimental period. The increase in concentration of α GP, lactate and malate was moderately higher in the ventricular than in the atrial muscle. The lactate/pyruvate and *α GP/DAP ratios increased to clearly higher values in the ventricular* than in the atrial tissue.

Effects of hypoxia and recovery from hypoxia on the concentrations of creatine phosphate and adenine nucleotides in the right atrial and the right ventricular muscle

The tissue concentrations of CP and adenine nucleotides in the right atrial and the right ventricular muscle found in the control group are presented in Table II. The concentrations of CP, ATP and ADP were significantly higher in the ventricular than in the atrial muscle, whereas the concentration of AMP did not differ significantly between the tissues. The sum of the concentrations of adenine nucleotides was significantly higher in the ventricular than in the atrial muscle.

During the period of hypoxia there was a decrease in the concentrations of CP and ATP and an increase in the concentrations of ADP and AMP in the atrial as well as the ventricular muscle (see Fig 4) These changes were reversed and the concentrations were restored approximately to the control levels during the recovery period

Regarding the kinetics of the changes in the concentrations of these compounds no essential difference was observed between the atrial and the ventricular muscle with respect to ATP and ADP The concentration of AMP however increased after 4 min of hypoxia to about 120 % of the control value in the ventricular muscle but merely to about 55 % of the control value in the atrial muscle Furthermore the ventricular concentration of CP decreased to a larger extent during the beginning of the experimental period and increased to a larger extent during the end of the period in comparison to the atrial concentration

During the period of the hypoxia recovery cycle when the abbreviations of the atrial and the ventricular MAPDs were recorded (*cf* Samuelsson 1973) the concentrations of CP were markedly reduced maximally to levels below 10 % of the control values after 4 min of hypoxia The concentrations of ATP showed more moderate reductions maximally to levels at about 70 % of the control values after 4 min of hypoxia

Discussion

The control values of the concentrations of CP and adenine nucleotides found in this study are in agreement with previous findings reported in the literature (Krause and Wollenberger 1965 Braasch *et al* 1968) as also are the concentrations of the glycolytic metabolites and malate (Klarwein Lamprecht and Lohmann 1962 Nagle Hockerts and Bogelmann 1963 Kubler 1970)

The findings of higher concentrations of CP ATP and ADP in the ventricular than in the atrial muscle confirm the results of Davies Francis and Stoner (1947) and Mulder Omachi and Rebar (1956) who in different mammals including the dog also found a greater storage of these compounds in the ventricular than in the atrial tissue This however does not necessarily imply a difference in the composition or the function of the different muscle cells but may be due to a different admixture of connective tissue or a different magnitude of the extracellular spaces between the tissues (*cf* Mulder Omachi and Rebar 1956) This reservation is supported by the reports of Danielson (1964) and Barclay Hamlay and Houghton (1959) who in the frog and rat hearts respectively found markedly higher values of the extracellular space in the atrial than in the ventricular muscle Thus further knowledge about the composition of the various tissues is required before definite conclusions regarding the cellular contents of these compounds can be drawn

The kinetics of the changes in concentration of ATP and ADP were found to be largely similar in the atrial and ventricular tissues With respect to CP however a more pronounced change in concentration was found in the ventricular than in

the atrial muscle during the early and late parts of the hypoxia recovery cycle The increase and decrease in the rate of anaerobic glycolysis as reflected in the change of the amount of lactate *a* GP and malate were found to proceed more rapidly in the ventricular tissue thereby indicating a higher rate of energy metabolism than in the atrial tissue This may be explained by the finding of a more rapid change in the concentration of AMP acting as a regulator of the glycolytic flow (Newsholme 1970) in the ventricular than in the atrial muscle

The continuing increase observed in the concentrations of malate during the early part of the recovery period may reflect an increased availability of oxaloacetate acting as a proton acceptor This may be due to reactivation of the citric acid cycle taking place during the recovery period

Recently McDonald Hunter and MacLeod (1971) have found in experiments performed on papillary muscle of the guinea pig heart that the regulation of the action potential duration is correlated rather to a certain portion than to the total tissue content of ATP For practical reasons however certain portions or pools of CP or ATP cannot be separately analyzed Conclusions regarding the supply of high energy phosphate compounds of different basic mechanisms of the cardiac cells thus have to be made from the total tissue concentrations

As found in this investigation the tissue content of ATP was reduced merely to about 70 % of the control values whereas CP was reduced to about 10 % of the control values during the period of 4 min of hypoxia which was long enough to induce a pronounced shortening of the atrial as well as the ventricular MAPD (*cf* Samuelsson 1972) Out of this is concluded that the total tissue content of CP may constitute a more sensitive measure than the total tissue content of ATP of the state of energy supply of the mechanism regulating the duration of the trans membrane action potential of heart muscle cells

The increase in the arterial plasma concentration of potassium found during the hypoxic period is in agreement with the results reported by Sybers Helmer and Murphy (1971) who reported a net loss of potassium from the heart during systemic hypoxia Thus the existence of a temporal correlation between the shortening of the action potential duration and the loss of potassium from the heart cells during hypoxia which has been reported by many authors (*cf* Hoffman and Cranefield 1960 Fenton Gudbjarnson and Bing 1966) is also implied by this work

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Effects of Severe Systemic Hypoxia on Myocardial Excitation

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Abstract

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In anesthetized dogs monophasic action potentials from the right atrium and the right ventricle were recorded by an intracardiac suction electrode technique together with the electrocardiogram (standard lead II) The animals were subjected by ventilation with 100 % nitrogen to periods of severe systemic hypoxia. The separate effects of myocardial oxygen deficiency and increased autonomic influence on the myocardial excitation were studied using animals with (1) intact autonomic innervation of the heart (2) parasympathetically denervated hearts and (3) parasympathetically and sympathetically denervated hearts During severe systemic hypoxia the parasympathetic influence predominated over the sympathetic and induced a pronounced shortening of the duration of the atrial monophasic action potential whereas the increased sympathetic activity induced no alteration of the duration of the ventricular mono phasic action potential The myocardial oxygen deficiency induced a shortening of the duration of the atrial monophasic action potential which appeared earlier than that of the duration of the ventricular monophasic action potential when the autonomic influences were excluded During the recovery from myocardial oxygen deficiency the duration of the atrial monophasic action potential was normalized earlier than that of the ventricular monophasic action potential Pronounced ST segment and T wave elevations in the ECG were in general recorded when the period of severe systemic hypoxia exceeded 2 min

It is well known that an inadequate oxygen supply accelerates the repolarization course of the transmembrane action potential of heart muscle cells (Trautwein Gottstein and Dudel 1954 Webb and Hollander 1956) For this reason regional im pairment of the myocardial circulation as in infarction gives rise to an increased temporal dispersion of the recovery of excitability in the heart muscle which favours the incidence of development of arrhythmias (*cf* Watanabe and Dreifus 1968) The significance of this matter has been established in both clinical and experimental work by many authors (for review see Szekeres and Papp 1971)

The arrhythmogenic influence exerted by increased autonomic discharge in the heart muscle has been well known for a long time (*cf* Szekeres and Papp 1971) Alessi *et al* (1958) and Han and Moe (1964) established that increased para sympathetic and sympathetic discharges cause a non uniform shortening of the re fractory periods which increases the asynchrony of the recovery of excitability in the atrial and ventricular muscles respectively

According to Watanabe and Dreifus (1968) and Vaughan Williams (1970) anything that shortens the absolute refractory period of heart muscle cells favours the development of re excitation and the induction of arrhythmias

The duration of the monophasic action potential recorded from the heart muscle by suction electrode technique is a good approximation to the duration of the action potential recorded by microelectrode technique in single heart cells in the immediate vicinity of the suction electrode (Hoffman *et al* 1959 Churney and Oshima 1964) and constitutes a valuable index for the determination of the refractory period (Olsson 1971)

The aim of this communication is to present studies of the influence of oxygen deficiency and increased autonomic discharge induced by severe systemic hypoxia (Downing Mitchell and Wallace 1963 Litwin and Skolasinska 1966) on the duration of atrial and ventricular monophasic action potentials recorded in the dog's heart *in situ* out from an arrhythmogenic point of view The intracardiac suction electrode technique used for this purpose has been described in a previous work (Samuelsson and Sjostrand 1971)

Methods and experimental procedure

The experiments were performed on mongrel dogs of both sexes weighing between 14 and 26 kg After the induction of anaesthesia with pentobarbital (Nembutal® 25 mg/kg) the dog was intubated and given artifical respiration (air) with high frequency positive pressure ventilation (Jonzon *et al* 1971) Maintenance doses of Nembutal on the average 50 mg/h were administered throughout the experiment

The thorax was opened by splitting the sternum and an incision approximately 10 cm long was made in the pericardium above the right atrial appendage and the right ventricular wall The artificial respiration was regulated after the thorax had been opened so that the arterial pO_2 was greater than 100 mm Hg and the acid base status was within the following limits pH 7 40 ± 0 05 pCO_2 40 ± 5 mm Hg

The heart was denervated parasympathetically by vagal division in combination with an i v administration of atropine sulphate (2 mg/kg) Sympathetic denervation of the heart was carried out by destroying the stellatum ganglias in combination with an i v administration of propranolol (Inderal® 1 mg/kg) During parasympathetic stimulation the vagus nerves were electrically stimulated (amplitude 10 V duration 2 ms frequency 20 Hz) with bipolar silver electrodes which were placed around the peripheral stump of the divided nerve During sympathetic stimulation the stellatum ganglias were electrically stimulated (amplitude 10 V duration 2 ms frequency 20 Hz) with bipolar silver electrodes which were hooked around the ganglias Severe systemic hypoxia was induced by exchanging the air in the ventilation system for 100 % nitrogen During the recovery phase the animal was again ventilated with air The changes in arterial pO_2 pH and the arterial plasma sodium and potassium concentrations induced by this type of hypoxia have been described by Fellenius and Samuelsson (1972) The animals were exposed to a maximum of [illegible] periods of hypoxia the first of which was never longer than 2 min

For the measurement of the arterial pH and pO_2 an Acid Base Analyzer Type PHM 71 connected to a Blood Micro System Type BMS [illegible] and a pO_2 electrode Type E 5046 (Radiometer Copenhagen Denmark) were used The pCO_2 was determined by means of the Siggaard Andersen Curve Nomogram for human blood using the Astrup technique (Siggaard Andersen 1967) A pacemaker catheter (USCI No [illegible]) localized in the upper part of the right atrium or in the apical region of the right ventricle was used to keep the heart rate constant and thereby to avoid variations in the monophasic action potential duration due to changes in the heart rate The monophasic action potentials were recorded together with the ECG (standard lead II) on an oscilloscope (Tektronix Type 561) and filmed with an oscilloscope recording camera Model C4 (Grass Instruments Mass USA) The monophasic action potentials were recorded from the right atrial appendage and/or from the apical region of the right ventricle The positions of the catheter tips were established by palpation through the pericardial incision

The duration of the monophasic action potential was measured at the level of 80 % repolarization which is a proper level when taken as an index of the refractory period (Olsson 1971) and is given as a percentage of the duration of the control recording (Table I). Qualitative changes in the duration of the monophasic action potential, the changes in the atrioventricular (A-V) conduction time and the ST segment and T wave changes in the ECG were determined by superimposing the control recordings on the experimental recordings. The time interval between the depolarization phases of the auricular and ventricular MAPs was considered to be largely a measure of the A-V conduction time (J. Bagdonas *et al.* 1961).

Results

Autonomic nerve stimulation

Experiments with electrical stimulation of the vagus nerves were performed on four dogs with sympathetically denervated hearts and experiments with electrical stimulation of the stellatum ganglias were performed on four dogs with parasympathetically denervated hearts. The experiments were carried out partly with spontaneous heart rate and partly with constant heart rate which was induced by a pacemaker catheter placed in the upper part of the right atrium. The ECG, the right atrial and the right ventricular monophasic action potentials were recorded during the experiments.

During the *stimulation of the vagus nerves* a strongly marked reduction of the heart rate was recorded (see Fig. 1A), which in most cases turned into complete sinus arrest. The shape of the atrial monophasic action potential was altered as a consequence of the acceleration of the repolarization course, which led to a strongly marked shortening of the duration. The duration of the ventricular monophasic action potential was increased in connection to the lowering of the heart rate. During paced constant heart rate, the same changes were recorded in the atrial monophasic action potential, but the duration of the ventricular monophasic action potential remained constant in this case (see Fig. 1B). The A-V conduction time was always increased and various degrees of A-V blocks were recorded during the stimulation (see Fig. 1B).

During the *stimulation of the stellatum ganglias* a pronounced increase of the heart rate and a reduction of the A-V conduction time were recorded in all cases (see Fig. 2A). The heart rate increase was measured as on the average 49 ± 9 % (Mean ± S.D.) of the initial rate. In most cases there was, both in the atrial and the ventricular monophasic action potentials, a slight increase in the plateau amplitude recorded during the sympathetic stimulation. This alteration was during paced constant heart rate accompanied in some cases by a minute increase and in some cases by a minute reduction of the monophasic action potential duration (see 2B). The alterations of the monophasic action potential duration recorded were as a general very small and in no case exceeded 3 % of the duration of the control recording.

Severe systemic hypoxia with spontaneous heart rate

These experiments were carried out on dogs with (1) intact autonomic innervation

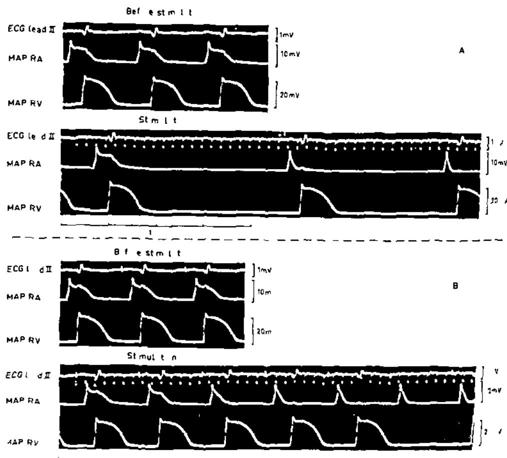


Fig 1 *ECG and endocardial recordings of atrial (MAP RA) and ventricular (MAP RV)* monophasic action potentials A Recordings before and during stimulation of the vagal nerves with a spontaneously beating heart B Recordings before and during stimulation of the vagal nerves with a paced constant heart rate

of the heart (2) parasympathetically denervated hearts and (3) parasympathetically and sympathetically denervated hearts Four dogs were included in each experimental series The dogs were exposed to severe systemic hypoxias for periods of 2 min (series 1 and 2) and 4 min (series 3) The ECG the right atrial and the right *ventricular monophasic action potentials were recorded before* and at every 15th second during the experimental period

In the experiments on dogs with *intact autonomic innervation of the heart* a pronounced decrease of the heart rate was recorded in all cases during the period of hypoxia which as a rule was observable even after 30 s of hypoxia The A V conduction time increased and a marked shortening of the duration ensued in the atrial monophasic action potential while the duration of the ventricular mono

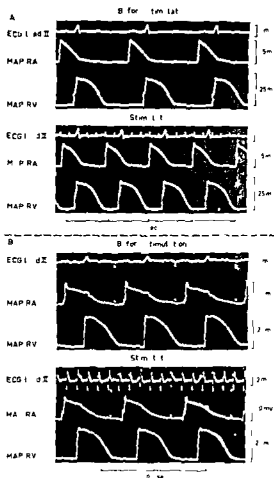


Fig 2 ECG atrial and ventricular monophasic action potentials A Recorded before and during stimulation of the stellatum ganglias with a spontaneously beating heart B Recorded before and during stimulation of the stellatum ganglias with a paced constant heart rate Control recordings have been superimposed on the atrial and ventricular recordings (bottom right) obtained during the stimulation of the stellatum ganglias

phasic action potential increased with decreasing heart rate (see Fig 3) The heart rate fell to between 24—39 % of the control rate during the experimental period These changes were in all cases in agreement with those recorded during the stimulation of the vagal nerves (cf Fig 1 A)

In the experiments on dogs with *parasympathetically denervated hearts* an increase of the heart rate was recorded in all cases during the experimental period which also as a rule was observable even after 30 s of hypoxia After 2 min of hypoxia the increase turned into a progressive decrease of the heart rate The A V conduction time was reduced in connection with the increase in the heart rate (see Fig 3) which in these experiments amounted to between 14—20 % of the control rate The changes recorded during the first 2 min of hypoxia were in all cases in agreement with those recorded during the stimulation of the stellatum ganglias (cf Fig 2 A)

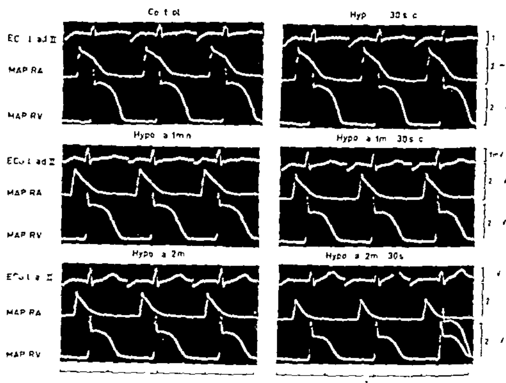


Fig 5 ECG atrial and ventricular monophasic action potential recordings during severe systemic hypoxia in a dog with a parasympathetically denervated heart at a paced constant heart rate A ventricular control recording has been superimposed on the ventricular recording obtained in hypoxia for 2 min 30 s

in the control recording No change was recorded in the duration of the ventricular monophasic action potential In some cases a second degree A V block arose during the later part of the period of hypoxia (see Fig 4)

Experiments with *parasympathetic denervation of the heart* were performed on four dogs During the first 2 min of the period of hypoxia a reduction of the A V conduction time was recorded which after 2—3 min turned into a progressive lengthening (see Fig 5) After 1—2 min of hypoxia a progressive shortening of the duration of the atrial monophasic action potential was recorded A corresponding change in the ventricular monophasic action potential was recorded only after 2—3 min of hypoxia

Experiments with *parasympathetic and sympathetic denervation of the heart* were performed on five dogs As in the experiments with parasympathetic denervation of the heart a progressive shortening of the duration of the atrial monophasic action potential was recorded after 1—2 min of hypoxia with the corresponding change in the ventricular monophasic potential ensuing after 2—3 min (see Fig 6) In these experiments as in the experiments with parasympathetic denervation of the

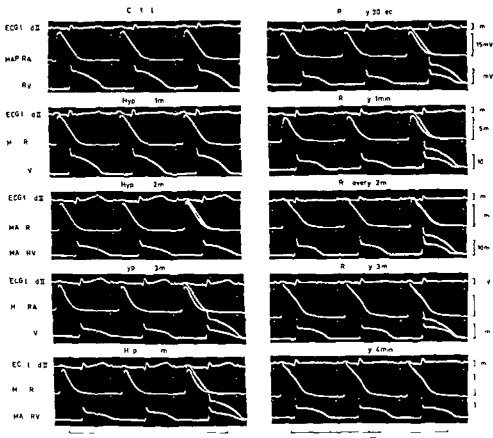


Fig 6 ECG atrial and ventricular monophasic action potentials from a dog with parasympathetically and sympathetically denervated heart during a period of 4 min of severe systemic hypoxia immediately followed by a period of 4 min of recovery at a paced constant heart rate Atrial and ventricular control recordings have been superimposed on recordings made during hypoxia

heart this shortening of the duration arose in all experiments earlier in the atrial than in the ventricular monophasic action potential After 2—3 min of hypoxia in all cases a progressive lengthening of the A V conduction time which during the first 2 min remained constant or was slightly reduced was recorded During the recovery period the duration of the atrial monophasic action potential was normalized within 1—2 min while the duration of the ventricular monophasic action potential was normalized only after 2—3 min (see Fig 6) In all cases the hypoxia induced shortening of the duration was normalized earlier in the atrial monophasic action potential than in the ventricular monophasic action potential

In order to make it possible to study the shortening of the duration of the ventricular monophasic action potential due to the hypoxia for a longer period than

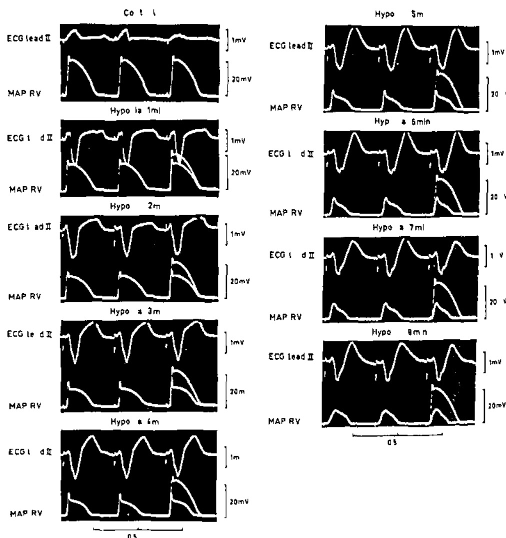


Fig 7 ECG and ventricular monophasic action potential recorded from a dog with parasympathetically and sympathetically denervated heart during a period of 8 min of severe systemic hypoxia at a paced constant heart rate (pace-maker tip in the right ventricle) A control recording has been superimposed on the recordings made during hypoxia

4 min, independently of the A V block which as a general arose after about 5 min the pacemaker catheter tip was placed in the apical part of the right ventricle In this way, the ventricular rate could be kept constant in all cases for an 8 min period of hypoxia Such experiments were carried out on five dogs with *parasympathetic and sympathetic denervation of the heart* (see Fig 7) The degree of shortening of the duration of the ventricular monophasic action potential recorded throughout these experiments is presented in Table I

TABLE I Mean duration of the right ventricular monophasic action potential (MAP) recorded during a period of 8 min of severe systemic hypoxia. The heart rate was kept constant throughout the experiment. The values are based on five experiments and expressed as percentage of the duration of the control recording

Period of hypoxia (min)	MAP duration in per cent of duration of control recording ± S.D.
Control	100 ± 0
1	100 ± 0
2	100 ± 0
3	93.4 ± 6.7
4	87.8 ± 12.1
5	82.0 ± 11.2
6	76.8 ± 14.6
7	74.2 ± 12.3
8	71.4 ± 13.3

ST segment and T wave changes

ST and T changes were recorded in most experiments in which the period of hypoxia exceeded 2 minutes. In practically all the cases the changes consisted of ST and T elevations (see Fig. 5, 6 and 7). No systematic temporal relationship could be established between the commencement of the shortening of the duration in the right ventricular monophasic action potential and the appearance of ST and T changes in the ECG, but in most cases the ST and T changes appeared before the shortening of the duration was recorded in the right ventricular monophasic action potential. During the recovery phase the ST and T changes were completely reverted and as a rule this took place before the duration of the right ventricular monophasic action potential had been normalized (see Fig. 6).

Discussion

Autonomic nerve stimulation

The decrease of the heart rate, increase of the A-V conduction time and shortening of the duration of the atrial monophasic action potential recorded during the electrical stimulation of the vagus nerves are in agreement with findings previously reported in the literature (cf. Hoffman and Suckling 1953, Hoffman and Cranefield 1960). No change however of shape or duration of the ventricular monophasic action potential was recorded at paced constant heart rate indicating that the excitation process of ventricular muscle cells remains uninfluenced during increased parasympathetic discharge (cf. Samuelsson and Sjöstrand 1972).

The increase of the heart rate, reduction of the A-V conduction time and increase of the plateau amplitude in the atrial and ventricular monophasic action potentials which were recorded during the electric stimulation of the stellatum ganglias are in correspondance with what other authors have reported previously (cf. Szek... and Papp 1971). During paced constant heart rate not even a slight sho

of the duration of the atrial and ventricular monophasic action potentials (*cf* Trautwein 1963 Han *et al* 1964) but in some cases even a slight increase of the monophasic action potential duration was recorded (*cf* Brooks *et al* 1955) These alterations of the monophasic action potential durations never exceeded 3 % of the control duration and in many cases no alterations at all were recorded during the sympathetic stimulation The findings thus indicate that increased sympathetic discharge may induce both an increase and a reduction of the refractory periods of atrial as well as ventricular heart cells and that the alterations induced are minute

Severe systemic hypoxia

During the first 2 min of severe systemic hypoxia a considerable decrease of the heart rate increase of the A V conduction time and shortening of the duration of the atrial monophasic action potential were recorded in dogs with intact autonomic innervation of the heart During the corresponding period of hypoxia an increased heart rate and a reduced A V conduction time were recorded in dogs with parasympathetic denervation of the heart. These results show that severe systemic hypoxia induces a considerable increase of the parasympathetic as well as the sympathetic activity in the heart where the effects of the increased parasympathetic activity is predominant (*cf* Levy 1971) This results in an increased temporal dispersion of the recovery of excitability in the atria (Alessi *et al* 1958) and in the ventricles (Han and Moe 1964) which will favour the induction of reentry mechanisms and the development of arrhythmias

Thus the findings reported accord with the lowered atrial and ventricular fibril lation thresholds induced by the autonomic nervous activity during systemic hypoxia which were found by Szekeres and Papp (1971) and furthermore stress the significance of the autonomic nervous system with respect to the inducement and maintenance of arrhythmias during systemic hypoxia

The alterations recorded in the heart rate the A V conduction time and the monophasic action potential duration in dogs with parasympathetically and sympathetically denervated hearts are considered to be due to local myocardial oxygen deficiency The functional changes appearing after 2—3 min of systemic hypoxia when a progressive reduction of the heart rate and an increase of the A V conduction time arose were reversible if the period of hypoxia did not exceed 4 min In these dogs as in the dogs with parasympathetically denervated hearts the hypoxia induced shortening of the duration ensued earlier in the atrial monophasic action potential (1—2 min than in the ventricular monophasic action potential (2—3 min) at a constant heart rate The duration was also normalized earlier in the atrial monophasic action potential (1—2 min) than in the ventricular monophasic action potential (2—3 min during the recovery phase

ST segment and T wave changes

The results in this investigation concerning the ST segment and T wave elevations in the electrocardiogram recorded during severe systemic hypoxia contradict the

results reported by Warren, Saurbrey and Wandall (1963), who found only minor electrocardiographic alterations consisting of flattening of the T wave and occasional ST segment depressions in dogs exposed to prolonged severe hypoxia. This difference is not easy to explain and may be due to the fact that the breathing mixture used by these authors contained 4 % oxygen in contrast to the complete lack of oxygen in the breathing gas used in this investigation. The results of this investigation demonstrate, however, that pronounced elevation of the ST segment and the T wave in the electrocardiagram may be recorded not only during coronary occlusion but also during severe, diffuse myocardial hypoxia.

Implications

The magnitude of the temporal dispersion of the recovery of excitability which arises between well oxygenated heart muscle tissue and heart muscle tissue which is exposed to severe hypoxia, as for example during myocardial infarction, is well illustrated by the comparison between the control and the experimental recordings of the ventricular monophasic action potentials seen in Fig. 7. The abnormal potential difference which will ensue between adjacent heart muscle fibers with such a disparity between the action potential durations is apparent. As a consequence, the presence of an infarction area in the heart muscle, creating such an asynchrony of recovery of excitability, must be considered as a highly predisposing factor for initiation of re-excitation and development of arrhythmias.

The combined effects of hypoxia and increased autonomic discharge may, during myocardial infarction, cause a greater disparity in the recovery times than is induced merely by the oxygen deficiency, thereby further increasing the probability of the induction of arrhythmias. In the results reported here, from dogs with parasympathetically denervated heart at paced, constant heart rates, no change of the ventricular monophasic action potential duration was recorded during the first 2 min of hypoxia, in spite of an obvious increase of the sympathetic activity during that period. The increased vagal activity, however, induced a shortening of the atrial monophasic action potential duration, which during the first 2 min of hypoxia clearly exceeded that induced by mere oxygen deficiency (as recorded in dogs with parasympathetically and sympathetically denervated hearts). From this it is concluded that the additional shortening of the refractory period of ventricular heart cells in an infarction area, which may be induced by increased sympathetic discharge, is minute in comparison with the reduction of the refractory period induced by severe oxygen deficiency. Increased vagal discharge, however, may, during atrial infarction, interfere with and possibly enhance the disparity of the recovery times of excitability, thereby increasing the likelihood of reentry phenomena and the induction of arrhythmias.

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Acta physiol scand 1973 88 281—283

From the Brain Research Laboratory E Blocket University Hospital Lund Sweden

Regulatory Mechanisms Affecting Carbohydrate Substrates in the Brain in Hypercapnic Acidosis

By

JAROSLAVA FOLBERGROVA[1] and BO K SIESJO

Recent evidence indicates that the intracellular pH in the brain is under metabolic control Thus when rats are exposed to increased CO concentrations the intracellular pH (pH_i) changes much less than the physico chemical buffer capacity of the intracellular fluids would predict (Siesjo Folbergrova and MacMillan 1972) Simultaneously there are decrea es in the tissue concentrations of a number of metabolic acids (Folbergrova MacMillan and Siesjo 1972) One possible explanation for this is that the acidosis transiently impedes substrate flux through the glycolytic and oxidative pathways thereby leading to a consumption of metabolic acid distal to the pH sensitive regulatory steps Results obtained on other tissues than the brain indicate that the regulatory enzyme phosphofructokinase (PFK) is sensitive to pH and that a pH activation of PFK is responsible for an increased glycolytic rate under alkalotic conditions The present experiments were undertaken to study the influence of hypercapnic acidosis upon levels of substrates in the glycolytic sequence and the citric acid cycle of the rat brain The objective of the study was to delineate possible regulatory steps responsible for the decreases in the steady state contents of the metabolic acids

Methods

The experiments were p rformed on male Wi tar rats (330—400 g) which were maintained paralyzed and artificially ventilated on 0 6—0 7 halothane and 30 O Hyper apnia was induced for 5 15 45 90 and 180 min by the adm n stration of ufficient CO_2 (11) to give an arterial P_{CO} of 85—90 mm Hg At the end of the e periment the tissue was frozen in s tu for subsequent analyses of metabolites (see Si jo *et al* 1972) The metabol te er measured with the fluorometric enzym c t chniques described by Lowry and Passonneau (197?) after extract on of the tissue at −2? C Since the ox loacetate oncentrations we too lo to allow accu ate analyses they were al ulated from the a partate am notransferase equilibrium using an equil brium constant of 6 7

Results

The figure shows the percentage changes in the concentrations of glucose (G) glucose 6 phosphate (G 6 P) fructose 1 6 diphosphate (FDP) dihydroxyacetone phosphate (DHAP) 3 phosphoglycerate (3 PG) pyruvate citrate α ketoglutarate

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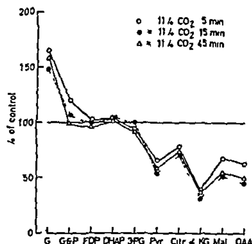


Fig 1 Influence of hypercapnic acidosis (11 % CO_2) upon brain tissue concentrations of glycolytic and citric acid cycle metabolites in brain tissue The values which are given as percentages of control values are the means for groups of 6 animals For abbreviations see text

malate and oxaloacetate (OAA) in hypercapnia (11 % CO_2) of 5 15 and 45 min duration At 5 min the glycogen concentration had decreased by 1 2 mmol/kg (from 3 45 ± 0 11 to 2 26 ± 0 09 mmol/kg), but in spite of this there was no significant increase in G 6 P concentrations The hypercapnia did not change the tissue concentrations of FDP DHAP or 3 PG At all exposure periods studied there were consistent decreases in the concentrations of all measured acids from pyruvate and onwards the pattern of change being very similar to that previously described (Folbergrova *et al* 1972)

Discussion

The evaluation of regulatory sites from changes in substrate levels is usually either based on the crossover theorem of CHANCE or on the postulate that the concentration of the substrate of a regulatory enzyme changes in the opposite direction to that of the flux (Krebs 1957) According to the crossover theorem a control site under the conditions of a decrease in flux is identified by an accumulation depletion crossover point It is not known if the glycolytic flux is changed in hypercapnia but we will assume that the experimental situation leads to a decrease in flux at least transiently

The present results show no changes in G 6 P or in FDP at any exposure time Thus the data do not support the hypothesis that the relative depletion of metabolic acids in moderate hypercapnia is due to inhibition of PFK At least part of the metabolic pattern seen at these degrees of hypercapnia could be due to loss of pyruvate and α ketoglutarate by means of CO_2 fixation (see Cheng 1972) However the results do not exclude the possibility that a regulatory influence on the levels of metabolic acids is exerted at the pyruvate kinase step

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Acta physiol scand 1973 88 284–286

From the Brain Research Laboratory E blocket University Hospital Lund Sweden

Homeostatic Regulation of Brain Energy Metabolism in Hypoxia

By

L D LEWIS U PONTEN and B K SIESJO

It has generally been assumed that the brain is markedly sensitive to hypoxia. Thus, consciousness is reported to be lost at a Pa_{O_2} of 30 mm Hg and calculations indicate that parts of the tissue become anoxic when the cerebral venous P_{O_2} is reduced to 17–19 mm Hg (for references see Siesjo and Plum 1973). However, recent results have shown that although lactate accumulates in the tissue below Pa_{O_2} values of about 50 mm Hg (Siesjo and Nilsson 1971) the arterial P_{O_2} can be reduced to 20 mm Hg and the venous P_{O_2} to 10–12 mm Hg before there are significant changes in the tissue concentrations of ATP, ADP or AMP (MacMillan and Siesjo 1972). The preservation of the cerebral energy state at these degrees of arterial and venous hypoxia, which requires that the mean arterial blood pressure is upheld at normal levels (Siesjo and Nilsson 1971), indicates the presence of efficient homeostatic mechanisms. One such mechanism is the increase in cerebral blood flow that occurs at low Pa_{O_2} values (Cohen *et al* 1967) and it has recently been suggested that the cerebral metabolic rate for oxygen is reduced (Duffy *et al* 1972). The present experiments had the objective of evaluating the ability of the tissue to maintain its energy state at degrees of hypoxia that lower the cerebral venous P_{O_2} to below 10 mm Hg and thereby to study the efficiency of the homeostatic mechanisms involved.

Methods

In rats that were anesthetized with 70% N_2O and artificially ventilated the Pa_{O_2} was reduced to 22–24 mm Hg for 15 min while the Pa_{CO_2} was maintained at 35–40 mm Hg. They were kept normothermic (37°C) and only those animals were included that had a mean arterial blood pressure of 1[illegible]0 mm Hg or more. There were two series. In one, arterial blood from a femoral artery and venous blood from the superior sagittal sinus were sampled at 2, 5 and 15 min of hypoxia for measurements of P_{O_2} and of total oxygen content (T_{O_2}). In the other series the brain was frozen *in situ* at either 2, 5 or 15 min for subsequent measurements of cortical concentrations of ATP, ADP and AMP using enzymatic fluorometric techniques.

Results

The figure shows that in the T_{O_2} series the arterial P_{O_2} was maintained at 22–24 mm Hg throughout the hypoxic period. However, since the plasma pH was gradually decreased from 7.40 ± 0.01 (mean ± S.E.) in the control group to 7.06 ± 0.01 in the

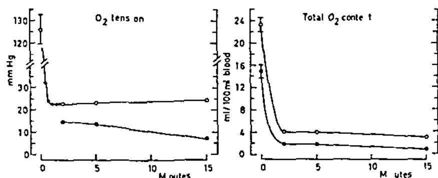


Fig 1 The oxygen tension and total oxygen content arterial (○) and superior sagittal sinus blood (●) during hypoxemia The values for 2—15 min are means for 6 animals with S E less than the size of the symbols The rate of decrease in arterial oxygen tension during the first 2 min (small dots) was determined in 2 animals by serial sampling

15 min group the arterial oxygen content fell from a value of 4 1±0 3 ml/100 ml at 2 min of hypoxia to 2 9±0 1 ml/100 ml at 15 min The gradual reduction of the arterial oxygen content was associated with a fall in the venous P_O that was 14 4±1 13 6±1 and 7 2±0 7 at 2 5 and 15 min respectively In spite of this fall in venous P_O the arteriovenous difference in oxygen content remained the same in the hypoxic groups In the 15 min group the oxygen saturation of the haemoglobin in the venous blood was reduced to 4 %

In the series used for analyses of tissue metabolites the Pa_{O_2} was 24 0±0 6 23 4±0 4 and 24 4±0 3 at 2 5 and 15 min of hypoxia respectively The table shows that the hypoxia did not significantly change the tissue concentrations of ATP ADP or AMP at any of these times and the calculated adenylate energy charge remained constant

Discussion

The present results corroborate our previous conclusion that the brain can withstand a pronounced reduction in arterial and cerebral venous P_{O_2} provided the blood pressure is upheld Thus the tissue can extract enough oxygen to maintain a normal energy state even if the arterial oxygen content is reduced from a normal value of

Table I Influence of hypoxia on cerebral cortex concentrations of ATP ADP and AMP and on the calculated adenylate energy charge (ECP) ECP = (ATP+1/2 ADP)/(ATP+ADP+AMP) Means±S E for groups of 6 animals

Group		ATP	ADP	AMP	ECP
Control		3 01±0 04	0 272±0 003	0 030±0 001	0 950±0 001
Hypoxia	2 min	2 99±0 03	0 278±0 006	0 028±0 002	0 949±0 001
	5 min	2 97±0 03	0 273±0 001	0 028±0 001	0 950±0 001
	15 min	2 99±0 03	0 285±0 007	0 030±0 001	0 948±0 001

about 23 to a value of 3 ml O /100 ml blood and even if the venous P_O is reduced to 7 mm Hg The a v differences in oxygen content suggest that this homeostatic main tenance of energy state is either caused by an increase in cortical blood flow to almost 400 % of normal or by a lesser increase in flow coupled with a corresponding reduc tion in metabolic rate The findings corroborate the conclusion that hypoxia in the absence of complicating ischemia does not readily disrupt the balance between production and utilization of energy in the brain (Siesjo and Plum 1973)

This study was supported by grants from the Swedish Medical Research Council (Projects No *14X 2179 and 14X 263) from the Swedish Bank Tercentenary Fund and by U S PHS Grant* No 5 R01 NS 07838 03 from NIH Dr Lewis is a recipient of a Swedish Medical Research Council postdoctoral research fellowship (Project No B73 60R 4076)

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Acta physiol scand 1973 88 287—288
From the Institute of Physiology University of Helsinki Finland

Responses Elicited by Intraventricular Injection of Carbachol in the Kitten

By

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There are few experimental data indicating the ontogenetic stages at which the neural mechanisms underlying agonistic (i e escape defensive threat and defensive attack) behaviour begin to function In the normal cat according to observations by Kling and Coustan (1964) most elements of this behaviour begin to appear spontaneously by 29—38 days of age However electrical stimulation of the hypothalamus and amygdala in kittens shows that mechanisms underlying at least some components of agonistic behaviour can function much earlier Chemical stimulation has not previously been used in studying the maturation of agonistic behaviour although it is well known that for example carbachol when injected into the hypothalamus amygdala hippocampus or third ventricle of the adult cat elicits a variety of responses (piloerection mydriasis defecation hissing attack etc) which are normal components of this behaviour (Baxter 1967 Desci Varszegi and Mehes 1969) In the present work we have studied the effects of this cholinergic drug on the behaviour of kittens of different ages

The material consisted of 11 kittens of ages varying from 1 to 20 days The weights of the kittens varied from 80 to 120 g The injections were made stereotactically into the third ventricle The co-ordinates for the ventricle in each case were calculated from the formaldehyde fixed head of a kitten of the same size from the same litter The injections were made under light halothane anesthesia (kittens older than 6 days) or after local infiltration of the skin with Lidocain® (1 to 6 days old) The quantity of carbachol injected into each kitten was 100 μg/kg b wt dissolved in 10 μl of saline For the observations on behaviour the kitten was placed on a table on which it was free to move about It was observed for 15 min before and 60 min after the injection Another kitten of the same litter was used as a control Into 5 of the control kittens we injected intraventricularly 10 μl of saline

Somatomotor responses were elicited by the injection of the drug even on the first day of life They included rotatory movements of the body and movements of the

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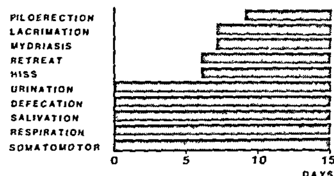


Fig. 1. Time table of the appearance of the responses elicited by intraventricular injection of carbachol in the kitten.

legs, head and jaws. The rotatory movements were usually observed after a time lag of 5 to 10 min and lasted for about 5 min. Effects on *respiration* were also observed on the first day of life. Immediately after the injection there was tachypnoea, which within about 10 min changed into bradypnoea. The frequent sniffing and panting observed were probably caused, at least in part, by the profuse *salivation* which occurred in all the kittens studied 5 to 10 min after the injection. In most kittens from the first day of life on, *defecation* and *urination* occurred within 10 min after the injection. On the sixth day of life *hissing* could be elicited after the injection in one animal by blowing into its face. Simultaneously, this kitten made movements resembling *retreat*. This behaviour occurred as much as 30 min after the injection. In the second youngest kitten (9 days old), in which hissing was observed, this response occurred after a time lag of 10 min. *Lacrimation* and *mydriasis* were observed from the 7th day onwards. In terms of age, the latest response to occur was *piloerection*. It was first observed in one 9 day-old kitten. The response occurred evenly all over body. Attacks or epileptic seizures were not evoked in these kittens. The time table of the appearance of the responses is summarized in the figure.

Except for occasional motor movements, the above described responses were not observed in the control kittens.

The results are in many respects in agreement with those of Kling and Coustan (1964), who used electrical stimulation of the hypothalamus and amygdala for evoking similar responses. However, some of the responses (i.e., salivation, defecation, urination, hissing and piloerection) elicited by carbachol in the present study appeared some days earlier than observed by these authors.

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Acta physiol scand 1973 88 289—295

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Vago vagal Gastro-gastric Relaxation in the Cat

By

HASSE ABRAHAMSSON and GUNNAR JANSSON

Received 11 July 1972

Abstract

ABRAHAMSSON H and G JANSSON *Vago vagal gastro gastric relaxation in the cat* Acta physiol scand 1973 *88* 289—295

In anesthetized cats electric afferent stimulation of nerve branches emanating from the gastric corpus region promptly elicited reflex gastric relaxation. The response was not blocked by atropine guanethidine or cervical spinal cord transection but was abolished by cervical vagotomy showing that the vagal relaxatory fibres to the stomach mediate this reflex effect Transient distension of the stomach produced a long lasting gastric relaxation with similar characteristics as that obtained by the mentioned afferent electric stimulation By vagal blockade in atropinized cats it was shown that the vagal nerves exerted no or only slight relaxatory effects if the stomach was only slightly filled but then had a profound relaxatory effect when the stomach was moderately or greatly filled It is concluded that a vago vagal gastro-gastric relaxatory reflex exists where the vagal non adrenergic relaxatory fibres serve as the efferent link The reflex can be activated from gastric mechanoreceptors responding to distension and is suggested to be involved in the regulation of receptive relaxation in gastric filling

It is well known that the stomach can receive considerable volumes with only slight increase in intragastric pressure (Kelling 1903 Grey 1918) though little is known about the mechanisms controlling this adaptation However recent studies in man (Aune 1969 Koster and Madsen 1970 Stadaas and Aune 1970) indicate that the vagal nerves may be important for maintaining the gastric pressure low when the intragastric volume is increased Martinson (1965) demonstrated the existence of a vagal non adrenergic fibre group producing pronounced gastric relaxation These relaxatory nerve fibres have been shown to form the efferent pathway in vago vagal reflexes elicited by afferent stimulation of vagal branches at the diaphragm level (Jansson 1968 1969 a Ohga *et al* 1969 1970) Furthermore they are also reflexly activated by esophageal distension by mechanical pharyngeal stimulation and during swallowing (Abrahamsson and Jansson 1969) These reflexes elicited from pharynx and esophagus are thought to be important for receptive relaxation of the stomach during food intake

The present study was performed to see whether the vagal relaxatory fibres to the stomach can be reflexly activated also from receptor mechanisms in the stomach

itself and whether such reflexes contribute to the remarkable ability of the stomach to adjust its volume to its contents.

Material and methods

17 cats weighing between 2.0 and 4.7 kg were used. They were deprived of food 24—36 h before the experiment. After induction with ether they were anesthetized with chloralose 40—65 mg/kg b.w. and a tracheal cannula was inserted.

Gastric motility was recorded as described previously (Jansson 1969 a). The *volume* of the stomach was recorded by means of a large rubber balloon *via* an esophageal catheter and a waterfilled volume reservoir connected to a float recorder. The reservoir functioning as an almost constant head of pressure was adjusted to keep the intragastric pressure at 5—8 cm H_2O except when the effect of distension of the stomach was studied (see below).

In 6 expts. nerve branches from the corpus of the stomach were electrically stimulated in the afferent direction. After midline abdominal incision 1—2 nerve branches along the lesser curvature or immediately distal to the gastroesophageal junction were carefully prepared free, cut and annular bipolar silver electrodes were placed around the central ends. Stimulations of 4—20 Hz, 5—8 V with a pulse duration of 0.5—2 ms were used.

Transient gastric distensions were performed by elevating the reservoir 5—15 cm above control level for 30 s to 2 min, thereby causing an inflow of fluid to the gastric balloon, after which the reservoir was reset to the predistension level. In 5 experiments the stomach was filled to different degrees by raising the reservoir 2 to 12 cm above the zero level (*cf.* Jansson 1969 a) and maintaining this level until volume adaptation was achieved.

For esophageal distension a rubber balloon, 5—7 cm long, was placed in the thoracic part of the esophagus. It was distended by 10—20 ml for 1/2—2 min (*cf.* Abrahamsson and Jansson 1969).

Vagal nervous activity could be blocked at cervical level either by nerve section or by local cooling with metal tubes perfused with a mixture of ice water and alcohol. In 2 expts. spinal cord transection was performed between C6 and C7.

Blood pressure was measured by a mercury manometer connected to the femoral artery. Atropine (Atropine sulphate Merck) was given i.v. in a dose of 0.5—1.0 mg/kg b.w. and guanethidine (Ismelin® CIBA) in a dose of 3—4 mg/kg b.w. In the spinal preparations artificial respiration was maintained by a respiration pump.

Results

Electric stimulation of the central end of cut nerve branches, emanating from the corpus of the stomach, elicited clearcut gastric relaxation (Fig. 1, panel A). The latency of this reflex response was 5—8 s. After the electric stimulation the gastric volume rather slowly decreased again, reaching control level first after 10—20 min. These reflex gastric relaxations persisted after blocking the excitatory cholinergic nerve supply to the stomach by atropine (Fig. 1, panel C), after administration of guanethidine to block the adrenergic fibres and after cervical spinal cord transection (Fig. 1, panel B). On the other hand, cutting the vagal nerves in the neck (Fig. 1, panel D) completely abolished the reflex gastric relaxation, indicating that it is of vago-vagal nature with the vagal non adrenergic relaxatory fibres forming *the efferent* link to the stomach (*cf.* Jansson 1969 a, Ohga *et al.* 1970).

To find out whether stimulation of mechanoreceptors in the stomach wall itself could activate the reflex described above, transient graded distensions of the stomach wall were performed in atropinized cats by elevation of the water reservoir allowing inflow into the gastric balloon. Fig. 2 illustrates an experiment in which such distensions ... 10 cm H_2O respectively are induced for

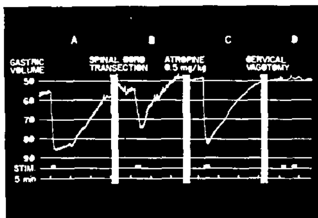


Fig 1 Cat 3 2 kg Gastric relaxation elicited by electric stimulation of the central end of a cut small nerve branch emanating from the body of the stomach The response persisted after spinal cord transection between vertebrae C6 and C7 (B) as well as after administration of atropine 0 5 mg/kg (C) but was abolished by section of the vagal nerves at cervical level (D) Nerve stimulation at 8 Hz 2 ms and 6 V Gastric volume given in ml

30 s With intact vagi (Fig 2 panel A) there is a rapid large increase of gastric volume during the distension Upon resetting the reservoir to the predistension level there is first a brief rapid phase of gastric volume decrease followed by a phase of slow accommodation to the predistension volume Blockade of the vagal nerves by cooling (Fig 2 panel B) changed the gastric response to such distension in two ways First the inflow to the stomach *during* the distension is now considerably reduced

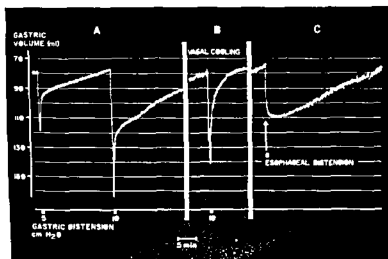


Fig 2 Cat 2 6 kg Atropine 0 6 mg/kg
A Gastric volume response to increase of 5 and 10 cm H_2O for 30 s in the applied gastric pressure Two-phasic regain in gastric volume upon resetting the pressure to control level
B Gastric response to an applied pressure increase of 10 cm H_2O for 30 s during cold blockade of the vagal nerve
C Gastric relaxation elicited by esophageal distension with 20 ml for 30 s Note the reduction in gastric volume increase to distension by blockade of the vagi and the similarity between the recovery phases after gastric distension (A) and esophageal distension (C)

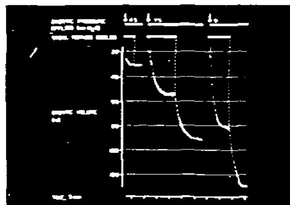


Fig 3 Cat 3.3 kg Atropine 1.0 mg/kg Gastric volume adaptation to different applied pressures during and after vagal cooling at cervical level. Note the gastric relaxation obtained upon warming the vagal nerves after cooling at pressures of 7.5 and 9 cm H_2O but not at 4.5 cm H_2O

(by 12—29 % in the different experiments) second the recovery phase *after* distension is now *characterized by a rapid return to predistension volume (Fig 2, panel B)* Thus the phase of slow long lasting regain of gastric volume after gastric distension (Fig 2 panel A) was essentially abolished by vagal blockade.

This type of long lasting gastric relaxation induced by gastric distension and vagally mediated was unaffected by administration of atropine and guanethidine. Further it closely resembled the reflex gastric relaxation induced by esophageal distension Fig 2 panel C) The results thus indicate that stimulation of mechanoreceptors by gastric wall distension elicits gastric relaxation through a vago-vagal reflex that utilizes the efferent vagal relaxatory fibres

The question arouses whether the vago-vagal reflex demonstrated above contributes to the adaptation of the stomach to its contents. In 5 cats the stomach was filled to different degrees by elevations of the water reservoir which was then kept at a constant level In the experiment illustrated in Fig 3 (atropinized cat) the extent of filling is shown when the stomach is subjected to inflow from three different reservoir levels first during vagal cold blockade and then when the vagal nerves are re-

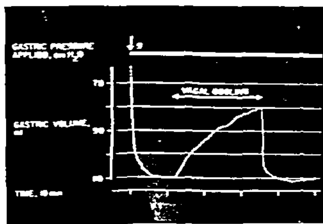


Fig 4 Cat 2.8 kg Atropine 0.8 mg kg Gastric volume decrease upon blockade of the vagal nerves by cooling when the stomach is filled and kept distended with a pressure load of 9 cm H_2O

warmed It is seen that this restitution of vagal transmission leads to considerable gastric relaxation at applied pressures of 7 5 and 9 cm H O but not at 4 5 cm H O In all experiments performed in this way the gastric relaxation dependent on vagal activity was absent or slight at low gastric pressures but at pressures of 6—7 cm H O or more the vagal relaxatory mechanism became pronounced and could cause a 50—90 % increase in gastric volume Furthermore as illustrated in Fig 4 when the stomach is filled and fully adapted to a certain volume with intact vagal nerves vagal cold blockade causes an immediate decrease of gastric volume increasing again upon rewarming of the vagal nerves to the level present before the cold blockade Also these responses indicate that the vagal relaxatory fibres take part in adjusting the stomach to its contents by mediating gastric relaxation as a result of afferent signals from mechanoroceptors in the gastric wall itself

Discussion

The present experiments demonstrate that vago vagal reflex relaxation of the stomach can be elicited from mechanoreceptors situated in the stomach itself and that such reflexes seem to play a role in adjusting the reservoir capacity during gastric filling

Afferent electric stimulation of cut nerve branches emanating from the stomach elicited gastric relaxation which persisted after cholinergic and adrenergic blockade and after cervical spinal cord section but was completely abolished by cervical vagal section Thus this reflex response showed the same characteristics as the vago-vagal gastric relaxatory reflex elicited by afferent vagal stimulation at the level of the diaphragm (Jansson 1969 a Ohga *et al* 1970) The efferent link of this reflex is constituted by the vagal non adrenergic relaxatory fibres described by Martinson (1965) and Campbell (1966)

Hence the present results indicate that these vagal fibres can be reflexly activated from the stomach itself thus constituting a vago-vagal gastro-gastric reflex They also indicate that this reflex at least in part emanates from mechanoreceptors in the gastric wall Thus brief periods of gastric distension leads to a long lasting reflex gastric relaxation which persists after adrenergic and cholinergic blockade It is however abolished by vagotomy and it closely mimics the recovery phase of the reflex gastric relaxation that can be elicited by esophageal distension (Abrahamsson and Jansson 1969) Further in steady state gastric pressure volume situations the vagal relaxatory fibres are active and contribute considerably to the relaxed state when the stomach is filled to higher degrees This is suggested by the fact that in atropinized cats with vagal cold blockade (Fig 3) rewarming of the vagal nerves leads to a pronounced gastric relaxation at higher gastric pressures while this is not the case at quite low gastric pressures The gastro-gastric vago vagal relaxatory reflex described above therefore seems to be activated not only d the dynamic phase of gastric distension but i continuously activated during

longed gastric distension keeping the gastric smooth muscles in a wellgraded state of relaxation whenever the stomach is filled to a moderate or high degree.

Mechanoreceptors in the stomach responding to distension are thus a prerequisite for eliciting the vago-vagal reflex described above. Electrophysiological recordings from vagal afferents have demonstrated gastric receptors responding to gastric inflation or to increase of tension in the gastric wall (Paintal 1954, Iggo 1957). These receptors are slowly adapting and considered to be *in series* with gastric smooth muscle cells thereby being capable to signal wall tension as well as gastric "fullness" (see Leek 1971). Such gastric receptors in non ruminant animals have been ascribed a role in the control of hunger and satiety (see Sharma 1967) but their importance for the regulation of gastric motility has been unclear. The present results suggest that such gastric receptors and their vagal afferents are important for the reflex adaptation of the stomach to its contents by activating the vagal relaxatory fibres.

The remarkable ability of the stomach to accomodate large volumes with only slight increases in intragastric pressure has been observed in both animals (Kelling 1903, Grey 1918) and man (Aune 1969). Gianturco (1934) showed that most of this receptive function occurred in the fundus and corpus of the stomach. Earlier studies on the role of the extrinsic nerves in the regulation of this gastric adaptation to increasing volumes have however given conflicting results (see *e.g.* Grey 1918) though recent pressure volume studies in man (Aune 1969, Köster and Madsen 1970) indicate that vagotomy leads to an increased tone of the stomach in contrast to widely hold views. The present results support such a view and reveal a reflex mechanism involved as is illustrated in *e.g.* Fig. 2 and 3. Therefore the present results strongly suggest that the mechanism by which the vagal nerves contribute to keep gastric tone low when the stomach is filled is at least in part constituted by a vago-vagal reflex activated from mechanoreceptors in the gastric wall with the vagal relaxatory fibres forming the efferent link. Vagotomy will of course interfere with this reflex mechanism thus enhancing gastric tone.

The vagal relaxatory fibres to the stomach seem to be of great importance for receptive relaxation of the stomach during food intake. Earlier experiments (Abrahamsson and Jansson 1969) strongly suggest that these fibres are reflexly activated already when food passes the pharynx and esophagus. The present results indicate that this receptive relaxation is also dependent on activation of mechanoreceptors in the stomach itself which contribute to the reflex excitation of the vagal relaxatory fibres.

When the effect of gastric distension was studied in the present experiments the same gastric balloon was used for distension as well as for recording of the effect of this distension. This technique had the advance that an increase in background discharge in different nerve fibre groups to the stomach (see Jansson 1969 b) induced by trauma to this organ could be avoided. However, to get a more detailed picture of the mechanism responsible for elicitation of vago-vagal gastro-gastric relaxation a pouch technique has been introduced and such investigations are in progress.

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1966) In the present study preliminarily reported (Abrahamsson Jansson and Martinson 1971) the effect of apomorphine on gastric motility was investigated with special reference to whether its action could be related to activity in any or both of these two nerve fibre groups to the stomach

Material and methods

17 cats weighing 2 0—4 6 kg were used deprived of food 24—36 h before the experiment After induction with ether free airway was secured by a tracheal cannula 8 cats were anesthetized with chloralose 40—60 mg/kg b w i v and 6 cats with urethane 1000—1200 mg/kg b w i v In 3 expts unanesthetized decerebrate cats placed on their side (cf Hesse 1913) were used In 2 of these ischemic decerebration was performed by ligation of the basilar artery and of the main carotids and their branches in the neck (after Pollock and Davis 1930) In one cat the cerebrum was removed by suction after opening of the skull

Gastric motility was recorded as described by Jansson (1969) A rubber balloon connected by a wide bore tube to a reservoir was placed in the stomach via the esophagus At low intra gastric pressures 5—8 cm H O the gastric volume was recorded by means of a float recorder writing on a smoke drum kymograph The volume of the emptied gastric balloon was about 15 ml which is included in values given in the figures

Apomorphine hydrochloride was administered either as i v infusion (8 expts) 0 2—30 mg/kg b w or directly into the right vertebral artery (9 expts) in doses of 0 1—30 mg/kg b w for details of the latter technique see Magni et al (1959)

Blockade of vagal activity was performed either by cutting the nerves in the neck or by cooling them with metal tubes perfused with a mixture of ice water and alcohol Electrical stimulation of the distal ends of the cut vagal nerves was performed with bipolar silver elec trodes Stimulations of 1—8 Hz 6—8 V and a pulse duration of 0 2—3 ms were used

Reflex activation of the adrenergic fibres to the stomach was induced by stimulation of mesenteric afferents (Jansson and Martinson 1966) The abdominal wall was then opened and the adrenals were ligated bilaterally The nerves around the superior mesenteric artery were cut and their central ends put into a ring electrode and stimulated with 10 Hz 2 ms and 6 V

Blood pressure was recorded by a mercury manometer connected to one of the femoral arteries Atropine (Atropine sulphate Merck) was given i v in a dose of 0 5—0 8 mg/kg b w and guanethidine (Ismelin® CIBA) i v in a dose of 3—5 mg/kg b w

Results

General appearance of gastric motility responses to administration of apomorphine In all cats apomorphine elicited a prompt and pronounced increase of gastric volume followed by a gradual slowly developing return to the initial volume as illustrated in Fig 1 A In some cats apomorphine produced beside gastric relaxation repeated forceful somatomotor retching movements which caused temporary reductions in gastric volume The latency for retching was 1 1/2—15 min but usually it began about 2 min (Fig 2) after administration of the drug The retching was always im mediately preceded by a phase of gastric relaxation (Fig 1 B)

The role of the vagal nerves in apomorphine induced gastric relaxation The gastric relaxation elicited by apomorphine persisted after administration of the adrenergic blocking drug guanethidine and after atropine blockade of the cholinergic excitatory fibres to the stomach The left panel of Fig 2 illustrates how apomorphine in an atropinized cat still produces a profound gastric relaxation Furthermore soon after the relaxation starts powerful retching temporarily empties the gastric balloon After cervical vagotomy (right panel) the gastric relaxatory response to apomo



Fig 1 Gastric relaxation induced by apomorphine in a chloralose anesthetized (A) and an unanesthetized decerebrate cat (B)
A Apomorphine administered into the right vertebral artery Note the rhythmic gastric contractions appearing also in the relaxed state
B Apomorphine given iv Gastric relaxation is observed about 10 s after infusion of the drug and furthermore just prior to retching which causes rapid temporary decreases of gastric volume

is completely abolished though the drug still produces retching movements This figure also shows that the fall in blood pressure generally observed after administration of apomorphine persists after cervical vagotomy and atropinization

As illustrated in Fig 3 (atropinized cat) a cold blockade of the vagal nerves also completely abolished the gastric relaxation response to apomorphine When however, the nerves some minutes later were rewarmed the stomach promptly relaxed to the volume earlier obtained with a similar apomorphine dose It is also seen that vagotomy performed when the stomach is in a relaxed state after apomorphine injection leads to a prompt decrease of gastric volume indicating interruption of an inhibitory vagal activity to the stomach After atropinization and vagotomy electric stimulation of the distal ends of the cut vagal nerves produced gastric relaxation (Fig 2 right panel) by activation of the vagal relaxatory fibres (*cf* Martinson 1965) showing that the gastric smooth muscles can still be relaxed when properly nervously influenced

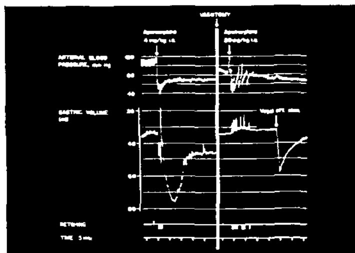


Fig 2 Cat 3 0 kg Unanesthetized decerebrate atropinized 0 5 mg/kg Apomorphine given into a vertebral artery
Left panel Depressor response gastric relaxation and retching induced by apomorphine
Right panel After bilateral cervical vagotomy the gastric relaxation to apomorphine is abolished but the depressor response persists and the retching movements still appear and cause short lasting decreases of gastric volume Gastric relaxation is however induced by stimulation of the distal ends of the divided vagal nerves (8 Hz 2 ms 6 V)

The role of splanchnic adrenergic fibres in apomorphine induced gastric relaxation Fig 4 illustrates how reflex activation of the splanchnic adrenergic fibres to the stomach by afferent mesenteric nerve stimulation promptly inhibits the excitatory gastric motor activity elicited by electrical stimulation of the distal ends of the cut vagal nerves (*cf* Jansson and Martinson 1966) On the other hand administration of apomorphine in a dose capable to elicit gastric relaxation when the vagal nerves



Fig 3 Cat 2 7 kg Chloralose 60 mg/kg atropine 0 7 mg/kg Gastric relaxation induced by i v administration of apomorphine The relaxatory response to apomorphine is absent when the vagi are blocked by cooling but upon nerve rewarming the stomach relaxes to the volume obtained after the first injection of the drug Note the decrease of gastric volume at the moment the vagal nerves are cut during the relaxed gastric state

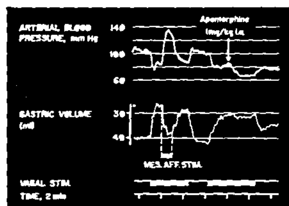


Fig 4 Cat 3 1 kg Chloralose 50 mg/kg vagal nerves cut with distal ends put on electrodes Reflex activation of splanchnic adrenergic fibres by stimulation of mesenteric afferents (10 Hz 2ms 6 V) promptly inhibits vagally elicited gastric contractions but apomorphine given under similar conditions has no clearcut effect on gastric motility Vagal stimulation at 2 Hz 3 ms 8 V

are intact has no clearcut effect on the stomach under these circumstances High concentrations of apomorphine in the CNS (8—10 mg/kg into the vertebral artery) slightly inhibited the vagally induced excitatory gastric activity This inhibition was however not comparable with the effect of afferent stimulation of mesenteric nerves

Importance of apomorphine dose and anesthetics In the present experiments the stomach was relaxed by much smaller doses of apomorphine than those required to evoke retching Thus, the lowest dose into the vertebral artery which induced retching was about 4 mg/kg b w (unanesthetized cats) while gastric relaxation was often produced by only 0 1 mg/kg i a or 0 2 mg/kg i v (anesthetized animals) The ng response to apomorphine was clearly affected by anesthetics since it was not ed in any of the chloralose anesthetized cats (apomorphine given ≤30 mg/kg) and only a few times in cats lightly anesthetized with urethane and then only after apomorphine doses ≥8 mg/kg i a

Discussion

The present study shows that parenteral administration of the drug apomorphine elicits profound relaxation of the stomach in the cat This gastric response preceded apomorphine induced somatomotor vomiting movements and was shown to be mediated by the vagal relaxatory fibres to the stomach since it persisted after administration of cholinergic and adrenergic blockers (Martinson 1965) but was abolished by vagotomy Furthermore vagotomy performed in atropinized cats when the stomach was relaxed by apomorphine administration led to a prompt decrease of gastric volume indicating an interruption of activity in the vagal relaxatory fibres

The findings that apomorphine had no effect on gastric motility after vagotomy whether gastric contractions were induced by electrical vagal stimulation or not suggest that splanchnic nerve fibres to the stomach (Jansson and Martinson 1966) were of little or no importance for the recorded gastric response to apomorphine These results also indicate that effects mediated *via* the adrenal medulla or caused by direct drug influences in the gastric wall were not essential for the gastric response recorded Further the fact that the gastric relaxatory response to apomorphine was

abolished after vagotomy excludes the possibility that gastric volume increase was secondary to changes in the tone of the abdominal skeletal muscles The sudden temporary decreases in gastric volume recorded in association with retching and vomiting movements obviously correspond to the expulsion of gastric contents ordinarily occurring during emesis as a result of the abdominal muscle contraction

Inhibitory influences of apomorphine on the stomach were observed in X ray studies in cats by Cannon (1898) He found that after subcutaneous administration of apomorphine a complete inhibition of the cardiac end of the stomach was the first change in the vomiting act In fact several other studies have shown that the proximal part of the stomach is dilated during vomiting both in animals and man (*e g* Hesse 1913 Lumsden and Holden 1969) The present results strongly indicate that this type of relaxation of the proximal part of the stomach is caused by an activation of the specific vagal relaxatory nerve fibres (Martinson 1965) Such a behaviour of the gastric smooth muscles seems purposeful since the gastric and intestinal contents that are to be expelled then tend to accumulate in the proximal part of the stomach before expulsion

Gastric emptying in man is considerably delayed by apomorphine given in doses too small to produce nausea or vomiting (Ramsbottom and Hunt 1970) In the present study the vagal relaxatory fibres to the stomach produced gastric relaxation after a dose of apomorphine only 1/40—1/100 of the lowest dose which produces retching and vomiting in the cat (*cf* Barnes and Etherington 1964) These data suggest that the vagal relaxatory fibres may also influence on gastric emptying rate They may do so by inhibiting the tone of the upper part of the stomach which according to Cannon (1898) acts as an active reservoir by pressing out its contents a little at a time to the antral pump

Whether apomorphine *via* the medullary centers influences the activity in the vagal cholinergic *excitatory* fibres to the stomach as well cannot be definitely answered at present since the vagal *relaxatory* fibres cannot be selectively blocked Rhythmic gastric contractions were sometimes recorded during the relaxed state after apomorphine administration (Fig 1 A) suggesting that cholinergic excitatory activity to the stomach was still present However gastric and duodenal motor events during vomiting are complicated (see *e g* Smith and Brizzee 1961 Lumsden and Holden 1969) and further studies are required to clarify the role of the different nerve fibre groups to the stomach in this act

Since the vagal relaxatory fibres to the stomach seem to be reflexly involved also in the receptive relaxation of the stomach during food intake (Abrahamsson and Jansson 1969) via pharyngeal and esophageal receptors it appears reasonable to conclude that this specific group of efferent vagal fibres modulates gastric reservoir function in both physiological and pathophysiological situations for the accumulation of food taken in and of gastric contents to be expelled when needed

This study was supported by grants from the Swedish Medical Research Council (B73 14X 16 09B B72-61P 3607 01 and B71 17X 3176-01) from Svenska Sallskapet for Medicinsk Forskning and from the Faculty of Medicine University of Goteborg

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Acta physiol scand 1972 88 303—316
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Effect of Histamine Loading on the Metabolism of ^{14}C-Histamine, Administered Intravenously or Orally, in Domestic Animals (Goat and Pig)

By

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Abstract

ELIASSEN K A *Effect of histamine loading on the metabolism of ^{14}C histamine administered intravenously or orally in domestic animals (goat and pig)* Acta physiol scand 1973 *88* 303—316

Isotope dilution technique has been used to examine the effect of histamine loading on the metabolism of orally and parenterally administered ^{14}C-histamine 3 % and 45 % of the radioactivity was recovered in the urine of goats and pigs respectively when only ^{14}C-histamine was given orally In goats the radioactivity was mostly present as unidentified metabolites In goats oral loading with 0 9—1 0 g histamine increased the fraction excreted as ImAA on an average from about 5 % to 34 % whereas the amount of unidentified metabolites was reduced Independent of the dose given about 30 % of orally administered radioactivity was excreted as ^{14}CO In pigs a moderate increase in ^{14} imidazoleacetic acid was observed after oral histamine loading In contrast to i v administered histamine orally administered histamine was partly excreted as ^{14}C-conjugated histamine in the pig In pigs and goats neither oral nor i v histamine loading seemed to have any effect on the fraction excreted in urine as ^{14}C-histamine The only significant effect on histamine catabolism of i v loading of histamine was a reduction of the fraction excreted as ^{14}C-1 4 MeHi. However such loading of histamine in pigs seems to liberate histamine and/or histamine metabolites since the increments in urinary histamine and histamine metabolites exceeded the administered dose

The metabolism of tracer doses of parenterally administered histamine is well studied in the most commonly used species of experimental animals but only very limited information is available as to the metabolism of large doses of histamine In experiments with mice Karjala Turnqvist and Schayer (1956) found that diamine oxidase plays a more important role in histamine metabolism when large doses of histamine are given than it does under physiological conditions One object of the present study was to examine whether such an effect of histamine loading on the catabolism of the substance could be demonstrated also in other species Goats and

Abbreviations Hi histamine 4(5) (β aminoethyl) imidazole 1 4 MeHi 1 4 methylhistamine 1 methyl-4 (β aminoethyl) imidazol ImAA imidazoleacetic acid imidazole-4(5) acetic acid 1 4 MeImAA 1 4 methylimidazoleacetic acid 1 methyl imidazole-4 acetic acid 1 5 MeImAA 1 5 methylimidazoleacetic acid 1 methyl imidazole 5 acetic acid PEG polyethylene gly

pigs two species which differ considerably regarding relative importance of the oxidative pathway for histamine degradation (Eliassen 1969 and 1971 b) were chosen for this purpose

Administration of histamine orally instead of parenterally has also been found to result in a relative increase in the oxidative catabolic pathway in mice (Schayer 1956) Since increased formation of histamine in the lumen of the gastrointestinal tract has been suggested to be of importance in the pathogenesis of some diseases in ruminants (Dougherty 1942 Dain Neal and Dougherty 1955) and in horses (Akerblom 1939) it was considered of interest to examine the metabolism of small as well as large doses of orally administered histamine

In sheep up to 30 % of the radioactivity of orally administered ^{14}C histamine has been found in the expired air as $^{14}CO_2$ (Sjaastad and Kay 1970) $^{14}CO_2$ in the expired air was therefore also estimated in the present study

Since higher spec act of CO_2 was observed in rumen content than in expired air, Sjaastad (1967) concluded that CO_2 was formed in the rumen Therefore the excretion of CO_2 in the expired air was in some cases compared with the radioactivity in the rumen liquor and in the blood plasma

Materials and Methods

Methods and reagents were partly the same as those previously described (Eliassen 1969 1971 a and b)

The methods used include determination of ^{14}C-metabolites by means of isotope dilution technique The histamine bio-activity of urine was estimated on isolated guinea pig ileum subsequent to ion exchange chromatography Conjugated Hi was determined as the increase in urinary free Hi which occurred on hydrolysis with 10 N HCl for 1 1/2 h MeImAA was estimated by the method of Granerus and Magnusson (1965) and Granerus (1968)

For pigs the recovery of histamine diphosphate and N acetylhistamine (believed to be identical to conjugated histamine Tabor and Mosettig 1949) (10—100 μg/10—100 ml) added to urine was 74 ± 9 S.D 5 expts) and 65 ± 14 (S.D 8 expts) respectively The corresponding values for goats were 78 % (75—81 % 3 expts) and 71 ± 10 % (S.D 6 expts) The recovery for 1 4 MeImAA hydrochloride (300—400 μg/100 ml added to goat or pig urine was 86 ± 20 % S.D 35 expts The values were corrected on this basis The values for 1 5-MeImAA were not corrected for analytical losses since this substance was not available in quantities sufficient for recovery experiments

Histamine 2 ring ^{14}C spec act 54 mCi/mmol was obtained from the Radiochemical Centre Amersham England 1 4 MeHi and 1 4 MeImAA were synthesized in collaboration with Maj Britt Johansson Klin Physiological Laboratory Lasarettet Lund Sweden Hydroxide of Hyamine® and the Insta-Gel® Emulsifier was obtained from Packard Instrument Company Inc. Illinois U.S.A The vein canulas Braunule® were obtained from B Braun Melsungen W Germany

Animals and feeding Three healthy female goats of Norwegian breed (two weighing 40 kg and one 50 kg) and two healthy pigs of the Norwegian "Land-swin breed one female (100 kg) and two castrated males 60 and 0 kg were used for the experiments The animals were housed in metabolism cages which permitted separate collection of urine and feces In some of the experiments with goats the urine bladder was catheterized Sufficient HCl to bring the pH of the urine below 2 was added to the collection bottles The goats were fed pelleted concentrates and hay The pigs were given concentrates Water was freely available throughout the experimental periods

Administration of Hi The purity of the injected ^{14}C Hi was checked by two dimensional paper chromatography followed by autoradiography Only one spot was detected The ^{14}C-Hi was therefore without further purification dissolved in 20 ml 0.9 % NaCl and administered to the animal. In pigs 50 and 90 μCi of ^{14}C-Hi were given intravenously and orally respectively Goats were given about 50 μCi regardless of route of administration One μCi corresponds to

2 06 μg Hi The amounts of non labelled histamine given are presented in the tables When the metabolism of intravenously administered Hi was examined the Hi solution was injected into the jugular vein in goats and into one of the ear veins in pigs Both species were given the orally administered Hi by way of a stomach tube A stomach tube was also used for the sampling of rumen liquor

Bleeding Blood samples were taken from the jugular vein into heparinized centrifuged tubes through a plastic canula Braunule® which was fixed to the animal before the start of the experiments To obtain plasma, the tubes were centrifuged immediately after sampling

Determination of radioactivity in blood plasma urine milk and rumen liquor For the determination of radioactivity in plasma, urine milk and rumen liquor 1 0 ml 100—500 μl, 1 0 ml and 100—250 μl of the respective fluids were suspended in 10 ml Instagel TM and counted in a Packard Tri Carb liquid scintillation spectrometer usually for 10 min

Determination of $^{14}CO_2$ $^{14}CO_2$ was determined by feeding the expired air into a suspension of 1 0 ml of 1 M hyamine® in 10 ml scintillation liquid Instagel TM Phenolphthalein was used as an indicator for the neutralization of hyamine® From titration of hyamine® alone 1 ml of the solution used was calculated to have the capacity to trap 1 mmol CO_2 Assuming that the expired air in goats contains about the same concentration of CO as in man namely 3 6 % (Guyton 1961 p 566) and using a volume expired per minute for goats of 5 7 1 (Spector 1961 p 267) it was calculated that a goat of average weight expires 9 2 mmol/min Thus 1 ml hyamine® would be equivalent to the CO_2 expired in 6 5 s

Results

Signs of intoxication could not be observed either in goats or in pigs when either small or gram doses of Hi were given orally This was also in the case when tracer doses of Hi were given intravenously but when mg doses were given i v toxic manifestations invariably appeared immediately and lasted for about half an hour Vasodilatation was the most usual sign of intoxication but spontaneous defecation vomiting and signs of headache were also observed in both species

Urinary excretion of Hi

The results are shown in Table I the values are given in terms of the base and they are corrected for analytical losses In most instances neither free nor conjugated Hi in the urine of goats could be determined by the methods used due to the presence of substances with antihistamine activity In urine of goats for which Hi values are given in Table I no antihistamine activity could be demonstrated In expts no 6 and 9 antihistamine activity was demonstrated in the assay of free Hi but not for conjugated Hi Table II shows the amounts in percentage of the administered histamine labelled and non labelled excreted with the urine as free Hi and conjugated Hi It should be noted that after i v administration of mg doses of Hi relatively much more nonlabelled conjugated Hi was found in the urine than the radioactive substance (Table II) In expt no 11 the increase in conjugated Hi corresponds to even more than the amount that was administered On the other hand after oral administration of gram doses of Hi only about 1/10 of what would be expected from the isotope dilution experiments was excreted as free Hi

1 4 MeImAA and 1 5 MeImA 4 The values for urinary MeImAA the first 24 h after administration of ^{14}C Hi are given in Table I The values for urinary (non radioactive) 1 4 MeImAA in the present study were only about 1/10 of those found in earlier experiments with pigs (Eliassen 1971 b) A possible explanation for this

TABLE I Urinary excretion of histamine 1 4 MeImAA and 1 5 MeImAA in goats and pigs given histamine intravenously and orally

Animal	Expt no	Non labelled Hi (mg) and way of adm	Histamine µg/24 h Total	Free	Conj	1 4 MeImAA mg/24 h	1 5 MeImAA mg/24 h	mol 1 4 MeImAA / mol Hi	C-1 4 MeImAA / C Hi	C 1 4 MeImAA / C-1 4 MeHi
Goat 4♂	1	0 i v	~ 0	–	–	0 8	1 0		30	70
Goat 4♀	2	4 i v	–*	–*	–	1 4	0		60	140
Goat 4♀	3	0 p o	–*	–*	–	3 0	0		4	
Goat 4♀	4	0 p o	28	–*	–	3 7	6 9	– (110)	4	3
Goat 4♀	5	910 p o	10 700	3?0	10 400	10	1 3	20 (0 7)	10	50
Goat 5♀	6	0 i v	5	–*	–	1 6	2 7	– (250)	30	70
Goat 6♂	7	5 i v	390	6 5	380	1 0	2 7	1?0 (2)	30	300
Goat 5♂	8	0 p o	11	–*	–	1 1	1 9	– (80)	?	8
Goat 5♀	9	1210 p o	9 700	410	9 300	22	3 2	40 (1 8)	7	440
Pig 6♂	10	0 i v	5 100	340	4 800	7 2	0	17 (1 1)	30	4
Pig 6♀	11	10 i v	18 600	500	18 100	35	0	55 (1 5)	50	10
Pig 6♀	12	0 p o	5 700	110	5 600	9	0	65 (1 3)	40	90
Pig 6♀	13	15?0 p o	50 700	490	50 200	168	0	270 (2 6)	30	80
Pig 9♀	14	0 i v	7 600	130	7 500	54	0	330 (5 6)	30	5
Pig 9	15	10 i v	14 100	170	13 900	70	0	330 (3 5)	70	9
Pig 9	16	0 p o	2 200	0	2 200	5	0	– (1 8)	30	180
Pig 9	17	1820 p o	64 500	460	64 000	275	0	470 (3 4)	40	70
Pig 14♀	41	–	360	32	330	12	trace	300 (29)		
Pig 14♂	42	–	415	34	380	10	trace	230 (21)		
Pig 14♀	43	10 i v	7 600	111	7 500	37	trace	260 (3 9)		
Pig 14♀	44	–	1 400	70	1 300	20	trace	230 (11)		

* Not measurable because of presence of antihistamine activity
( ) Tot l Hi used in the calculations
Hi and MeImAA are expressed as the base and acid respectively

is that the food did not contain antibiotics as it did in 1971 Antibiotics might alter the balance between the bacteria in the gastrointestinal tract resulting in a situation favouring Hi formation

Table II shows the amount of labelled and non labelled 1 4 MeImAA excreted the first 24 h after administration expressed as per cent of the administered Hi dose It should be noted that i v injection of mg doses of Hi resulted in an increased excretion of 1 4 MeImAA which corresponded to 2—3 times what was injected while no significant change in the excretion of 1 5 MeImAA could be seen The last mentioned component increases on injection of large doses of Hi to mice (Karjala and Turnquest 1955) The present findings however are in accordance with the results of Kelvin (1970) which indicate that 1 5 MeImAA is not a metabolite of Hi

Urinary excretion of ^{14}*C activity after administration of* ^{14}*C Hi* As shown in Table III the radioactivity recovered in urine was much lower after oral administra

TABLE II Urinary histamine metabol tes expressed as per cent of admini tered dose

Animal	Expt no	Amount histamine given (mg)	per cent of administered histamine excreted as					
			Conjugated Hi		Free Hi		1 4 MeImAA	
			non labelled	C-labelled	non labelled	' C-labelled	non labelled	' C-labelled
Goat 4	2	4 i v	—	< 0 09	—	0 3	—	17
Goat 4	5	910 p o	1.2	< 0 2	0 04	0 2	0 6	ᵃ
Goat 6	7	5 i v	7	< 0 06	0 1	0 5	—	14
Goat 5	9	1210 p o	0 8	2	0 03	0 4	1 3	ᵃ
Pig 6	11	10 i v	130	< 0 2	2	< 0 8	210	42
Pig 6	13	1570 p o	3	4	0 02	0 5	8	15
Pig 9	15	10 i v	90	< 0 3	0 5	0 9	320 (130)**	63
Pig 9	17	1870 p o	3	2	0 07	0 5	11	19
Pig 14	43	10 i v	72	—	0 8	—	205	—

The values for the non radioactive Hi conjugated Hi and 1 4 MeImAA are obtained by subtracting the mean excretion for the respect ve compound obta ned when only C-Hi was given from that obtained after Hi loading (Table I)
The mean values for the non rad oact ve metabolites n expts no 6 and 8 (Goat 5) Table I were subtracted from the values found in expt 7 with goat 6 when the recovery was calculated
This was because no experiment w th only C-Hi was done with goat 6
** Value obtained if only the value in expt 14 is used as control

TABLE III Excretion of radioact vity the first days after intravenous and oral administration of C-h stamin without and with histam ne carrier The results are expressed as per of administered dose

Animal	Expt no	Non labelled Hi (mg) and way of adm	Urine			Feces			Milk			Sum	CO	Sum
			day 1	day 2	day 3	day 1	day 2	day 3	day 1	day 2	day 3			
Goat 4	1	0 i	93	3 1	—	0 9	—	—	0 07	—	—	97	1	98
Goat 4	2	4 i v	92	—	—	—	—	—	0 04	—	—	97	3	95
Goat 4	3	0 p o	3 6	1 5	0 3	0 3	0 2	0 1	3 5	2 1	0 3	11 9	—	—
Goat 4	4	0 p o	1 6	0 3	0 1	1 8	2 2	0 9	3 5ᵇ	—	—	10 4	37	42
Goat 4	5	910 p o	19	5 4	0 3	0 9	1 3	—	1 0	—	—	28	22	50
Goat 5	6	0 v	84	—	—	—	—	—	—	—	—	84	—	—
Goat 6	7	5 i	65	3 6	2 7	0 6	0 3	0 2	—	—	—	72	8	80
Goat 5	8	0 p o	1 7	0 3	—	1 2	0 9	—	—	—	—	4 1	31	35
Goat 5	9	1210 p o	19	5 1	—	0 2	2 4	—	—	—	—	27	31	58
Pig 6	10	0 v	87	1 9	—	—	—	—	—	—	—	89		
Pig 6	11	10 i v	9	1 ?	—	—	—	—	—	—	—	80	—	
Pig 6	12	0 p o	4[illegible]	—	1 0	0 5	8 7	1 [illegible]	—	—	—	58		
Pig 6	13	1520 p o	4[illegible]	3 0	0 3	1 0	0 4	0 2	—	—	—	51		
Pig 9	14	0 i	80	1 6	0 9	—	—	—	—	—	—	83		
Pig 9	15	10 v	81	1 3	0 6	—	—	—	—	—	—	83		
Pig 9	16	0 p o	47	1 1	0 03	2	0 2	0 4	—	—	—	51		
Pig 9	17	1870 p o	46	0 6	0 3	2 1	? 5	0 9	—	—	—	52		

a C-H account d for about 1 5 of th r 1o ctiv ty
b C-H account d for abcut 0 05 of th rad o ct ty
c C-H account d for about 0 ? of th rad oact ty

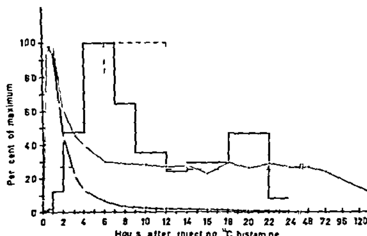


Fig 1 Excretion of radioactivity after oral administration of only ^{14}C-Hi to a goat. (Expt. no 4) Radioactivity in urine ——— milk ——— exhaled CO_2 ●———● and blood plasma +———+ All values are expressed in per cent of maximum radioactivity

tion than after i v injection Neither in goats nor in pigs could there be observed any significant difference between the amounts of radioactivity excreted after i v injection of ^{14}C histamine only and that excreted after ^{14}C Hi had been given together with large quantities of non labelled Hi The same held true for Hi administered orally to pigs but in goats a marked increase in renally excreted radioactivity was observed when carrier histamine was given together with ^{14}Ci Hi The pattern for the urinary excretion of ^{14}C activity is shown in Fig 1—4

Quantitation of ^{14}C Hi metabolites in the urine

The results of the isotope dilution analyses are shown in Table IV In goats i v injection of tracer doses of Hi gave somewhat lower values for the part excreted as total ImAA (free and riboside) than what was obtained in earlier experiments (Eliassen 1969) but apart from that the metabolic profile was about the same as in 1969) In pigs no differences could be seen between the metabolic pattern of i v injected Hi found in 1971 (Eliassen 1971 b) and that found in the present study

With the exception of a reduction in the part excreted as 1 4 MeHi in pigs i v loading with mg doses of Hi gave no significant change in the metabolic pattern either in pigs or in goats

In goats orally administered Hi seemed in contrast to i v administered Hi to a very low extent to be metabolized to ImAA Most of the urinary metabolites were unidentified metabolites The methylated metabolites accounted for about the same part of the urinary radioactivity as after i v injection

In pigs methylation seemed to play a less important role in degradation of orally administered Hi than of i v administered Hi The reduced methylation was compensated by an increased oxidative deamination to ImAA This was even more pro-

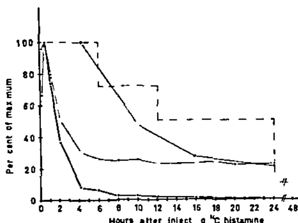


Fig 2 Excretion of radioactivity after oral administration of only ^{14}C-Hi to a goat (Expt nr 8) Radioactivity in urine ——— exhaled CO_2 ●———● blood plasma +———+ and rumen liquid O———O All values are expresed in per cent of maximum radioactivity

nounced under oral Hi loading In goats oral loading resulted in a markedly increased excretion of ImAA and a reduction of unidentified metabolites

Excretion of ^{14}C activity in feces expired air and milk after oral administration of ^{14}C Hi

Feces The radioactivity excreted with feces after oral administration of ^{14}C Hi was low (Table III) and of about the same order of magnitude as when ^{14}C Hi is given *i v* (Eliassen 1969 1971 b) Further regardless of route of administration no difference could be observed in fecal radioactivity in animals given only tracer doses of Hi and those loaded with Hi

Expiration air In goats the radioactivity expired as ^{14}CO during the first 24 h after oral *administration of ^{14}C Hi accounted for about 30 % of the administered* dose (Table III and Fig 1—4) Loading with Hi did not change the total amount of ^{14}CO excreted but the pattern of excretion was different from that found when only ^{14}C Hi was given (Fig 1—4) A dose dependent delay in the peak ^{14}CO on loading was observed The radioactivity of expired CO seemed to parallel the radio activity of the plasma The radioactivity in the expired air decreased however some what faster than the radioactivity in plasma The excretion of radioactivity in the expired air of goats after i v injection of ^{14}C Hi was very low about 10—20 times the background counts In pigs ^{14}CO in the expired air was not measured because of practical difficulties

Milk Many papers have reported the occurrence of high levels of Hi in milk (among others Eliassen 1971 a) After i v injection of ^{14}C Hi in cows only a tracer amount of the radioactivity was recovered in the milk After oral administration of ^{14}C Hi to goats in the present study only a very small part of the administered radioactivity could be accounted for in the urine It was therefore of interest to examine if orally administered Hi would increase the excretion of milk Hi Only a small part (~ 4 %) of the orally administered radioactivity was excreted with the milk the first

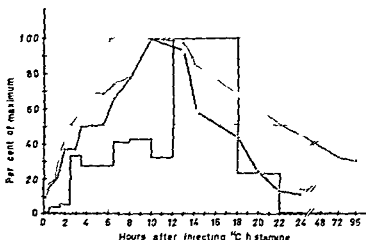


Fig 3 Excretion of radioactivity after oral administration of ^{14}C-Hi together with 0.01 g non labelled Hi to a goat (Expt no 5) Radioactivity in urine — — — milk ——— exhaled CO ●———● and blood plasma +———+ All values are expressed in per cent of maximum radioactivity

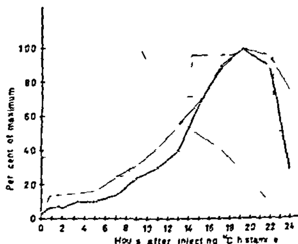


Fig 4 Excretion of radioactivity after oral administration of ^{14}C-Hi together with 1.2 g non labelled Hi to a goat Expt no ? Radioactivity in urine — — exhaled CO_2 ● — ● blood plasma and rumen liquid ○— ○ All values are expressed in per cent of maximum radioactivity

day after administration but this is relatively high compared with what was found after i v administration of Hi ~ 0 1 ,

As indicated in Table III only 0 0 to 1 of the radioactivity of the milk was accounted for by unchanged Hi This indicates that Hi fragments formed in the rumen (by rupture of the imidazole ring are more easily transferred to the milk than Hi and its ring metabolites After oral administration of tracer doses of ^{14}C-Hi to goats the radioactivity in milk and urine was of the same order of magnitude Under oral loading the radioactivity increased in urine but not in milk Patterns of excretion of radioactivity with milk are shown in Fig 1 and 3

TABLE IV Quantitative analysis of histamine metabolites in the first 24 h urine of goat and pig after administering ^{14}C-histamine together with carrier histamine i.v. or p.o.

Animal	Expt no	Non labelled Hi (mg) and way of adm	Excreted radioactivity pr 24 h in % of adm	Histamine metabolites urine Hi	of total ^{14}C in the first 24 h 1,4 MeHi	Total ImAA	1,4 MeImAA	Conjugated Hi	Sum of metabolites
Goat 4	1	0 i.v.	93	0.6	0.21	61	15	< 0.5	77
Goat 4	2	4 i.v.	92	0.3	0.13	54	18	< 0.1	72
Goat 5	6	0 i.v.	84	0.7	0.3[illegible]	60	22	< 0.1	83
Goat 6	7	5 i.v.	65	0.7	0.07	50	21	< 0.1	72
Goat 4	3	0 p.o.	3.6	4±2	4.6	3	14	< 1	26
Goat 4	5	910 p.o.	19	1	0.2	41	10	< 1	53
Goat 5	8	0 p.o.	1.7	1	0.2	6	1.5	< 1	9
Goat 5	9	1210 p.o.	19	2	0.03	26	13	10	50
Pig 6	10	0 i.v.	73	2	13.9	21	52	< 0.3	94
Pig 6	11	10 i.v.	79	1	5.6	24	53	< 0.3	84
Pig 9	14	0 i.v.	80	2	11.4	26	61	< 0.3	100
Pig 9	15	10 i.v.	86	1	7.9	20	73	< 0.3	102
Pig 6	12	0 p.o.	46	1	0.4	41	37	~ 3	82
Pig 6	13	1520 p.o.	46	1	0.4	55	33	~ 8	97
Pig 9	16	0 p.o.	47	2	0.4	29	70	~ 6	107
Pig 9	17	18 0 p.o.	46	1	0.6	48	42	~ 5	97

Unchanged ^{14}C-histamine and its main metabolites are expressed as per cent of the urinary excreated radioactivity.
The amount of carrier histamine is expressed as mg histamine base.

Radioactivity in rumen

The disappearance patterns for ^{14}C activity from rumen after oral administration of small and large doses of Hi to goat no. 5, expts. no. 8 and 9, are shown in Fig. 2 and 4 respectively. In expt. no. 8 the goat was given only ^{14}C Hi. After 4 1/2 h the first sample of rumen fluid was aspirated. At this time the remaining radioactivity in rumen was calculated to be about 10 % of the dose given. In expt. no. 9 the first rumen sample was taken 9 1/4 h after Hi loading. The remaining ^{14}C activity in the rumen at this time was calculated to be about 50 % of the administered dose. A rumen volume of 4 l was used for the calculations.

Analysis of the radioactivity in the rumen of the goat in expts. no. 8, 4 1/2 h after Hi was given, showed that about 12 % of the radioactivity was accounted for by unmetabolized Hi. Later experiments have shown that most of the rumen radioactivity 3—5 h after administration of ^{14}C Hi are due to ^{14}CO (Sjaastad and Eliassen, unpublished data). Analysis of the rumen content after i.v. administration of large doses of Hi (expt. no. 2) showed that if any of the radioactivity in rumen was due to unchanged Hi, it must be less than 0.3 %.

^{14}C activity in plasma after oral administration of ^{14}C Hi

The radioactivity in plasma during the experimental period in expts. no. 4, 8, 5 and 9 are shown in Fig. 1—4 respectively. The peak in the plasma radioactivity appeared all the earlier the less Hi given. At the time of peak radioactivity the total r...

activity in plasma was calculated to account for 1 5—3 % of the administered dose Experiments with Ba precipitation of CO in the plasma showed that only a small part if any of the radioactivity in the plasma could be accounted for by ^{14}C carbonate

Discussion

The metabolism of orally administered tracer amounts of ^{14}C Hi

The great difference between goats and pigs in the handling of dietary Hi which inter alia manifested itself as a marked difference in the urinary excretion of radioactivity and in the exhalation of $^{14}CO_2$ in goats but not in pigs is probably due to differences in digestion in the two species

The figures for the radioactivity excreted with the urine in pigs, after oral administration of ^{14}C Hi are similar to those found in man (Schayer 1956) in whom about 60 % of the administered dose was excreted with the urine The corresponding figures for goats on the other hand were more like those found for sheep (Sjaastad 1967)

When ^{14}C Hi was administered i v high levels of radioactivity were recorded in the urine in both goats and pigs (Eliassen 1969 1971 b and Table III of the present study) On this basis the lower urinary radioactivity found after oral administration of ^{14}C Hi might have been expected to be accompanied by higher levels of fecal radioactivity With the exception of one experiment on a pig (expt no 12) which developed diarrhea during the trial this was not the case The sum of radioactivity in urine feces and expired CO in the goats was of the same order of magnitude as e radioactivity recorded in urine of the pigs which amounted to less than 50 % of the administered dose Thus the elimination of radioactivity by way of ^{14}CO in the goats does not alone explain the low urinary and fecal radioactivity levels in this species

Since only a very small part of the radioactivity was recovered in the urine and since a considerable amount of radioactivity was expired as ^{14}CO in goats after oral administration of Hi the metabolites in urine give little information about the overall metabolism of orally administered Hi in this species To some extent this also seems to be the case in pigs since less than 50 % of the radioactivity given *per os* was recovered in the urine However the Hi metabolites found in the first 24 h urine after oral administration of Hi may be representative for the metabolites excreted with urine By comparing the Hi metabolites found in urine after i v injection of ^{14}C Hi with those found after oral administration it may be possible to reveal roughly what happens with orally administered Hi before it reaches the systemic circulation No final conclusion can however be drawn as to whether Hi is mainly absorbed from the gastrointestinal tract as Hi or its metabolites

In both pigs and goats the fraction excreted as unchanged ^{14}C Hi after oral administration was very low and corresponded well with that found after i v administration in the present study and with findings after oral administration in man (Granerus 1968)

The experiments in pigs indicate a tendency for orally given Hi to be relatively less methylated and more oxidatively deaminated to ImAA before reaching the systemic circulation than after This observation seems to agree well with the finding that the small intestinal mucosa in pigs mainly oxidizes Hi to ImAA (Eliassen in press) On the other hand in one of 2 expts with goats (expt no 4 Table IV) methylation seemed to be quantitatively more important than oxidative deamination which is the main metabolic pathway for i v injected Hi in this species (Eliassen 1969) This seems to agree well with results from the incubation of Hi with small intestinal mucosa of goats in which methylation plays a relatively important role (Eliassen in press) Schayer (1956) found that in cats and man methylation is the principal mode of metabolism for both fed and parenterally administered Hi Schayer's experiments with mice (1956) on the other hand gave results corresponding well with those obtained in experiments with pigs Mice methylate most of the injected Hi while orally administered Hi is mainly oxidatively deaminated The present findings and the findings of Schayer (1956) indicate that only in some species will oral and parenteral Hi administration give rise to differences in urinary Hi metabolites

The exhalation of considerable amounts of ^{14}CO in goats given ^{14}C Hi orally confirms previous results from experiments with another ruminant the sheep (Sjaastad and Kay 1970) demonstrating a considerable splitting of the imidazole ring and formation of ^{14}CO from ^{14}C-Hi given *per os* In a non ruminating species the pig only insignificant amounts of radioactivity can be accounted for in exhaled air (Sjaastad and Eliassen to be published) This indicates indirectly that the splitting of the imidazole ring probably takes place in the rumen a conclusion which Sjaastad and Kay (1970) had drawn from the fact that rumen CO had a higher spec act than exhaled CO

Nearly all the radioactivity in the pig urine was accounted for by known Hi metabolites while only about 20 % of the radioactivity in goat urine could be ascribed to known Hi metabolites In goat urine the remaining radioactivity probably represents fragments of the broken imidazole ring

Effect of oral Hi loading on the metabolism of fed ^{14}C Hi

The experiments involving oral loading of Hi revealed a discrepancy between the results of the biological and chemical assay of Hi in urine Before analyzing the effect of oral Hi loading on the metabolic pattern of fed ^{14}C-Hi possible reasons for this discrepancy will be discussed

In pigs the percentage increase in the urinary unchanged Hi estimated by biological technique occurring after oral administration of large Hi doses was much smaller than the fraction calculated by help of isotope dilution technique Since it is unlikely that labelled and non labelled Hi should be treated in different ways by the animal the above mentioned difference may be of methodological nature determination of Hi on isolated guinea pig ileum may in some instances result in too low values due to the presence of substances blocking contraction induced by Hi The presence of such substances would be difficult to reveal in urine containing la

amounts of Hi On the other hand ^{14}C Hi determined by isotope dilution technique may be overestimated because unknown Hi metabolites formed after oral administration of Hi may be difficult to remove from the Hi-crystals The fact that difficulties were often encountered in obtaining constant specific radioactivity of the Hi pipsyl chloride-crystals adds weight to such an assumption According to Granerus (1968) the values of ^{14}C-Hi could be falsely high if the urine contains some conjugated ^{14}C Hi because this compound might be partly hydrolyzed to free Hi when using the analytical procedure of Schayer A final conclusion with respect to the reasons for the observed discrepancy between the 2 methods can not be reached

In spite of the fact that the recorded ^{14}C Hi values may be too high the present results indicate that oral loading with up to 2 g Hi did not increase the percentage fraction of ^{14}C-Hi excreted unchanged in the urines either in goats or in pigs (Table II and IV) indicating that both species are well equipped to metabolize gram doses of orally fed Hi In contrast to findings in man to whom large doses of Hi have been given by mouth without any significant increase in urinary free Hi (Adam 1950 Mitchell and Code 1953 and Sjaastad 1966) a significant increase in the urinary Hi was observed on oral Hi loading in both pigs and goats (Table I) The increase was as indicated above less than what would have been expected from the radioisotope dilution assay of ^{14}C Hi The increased excretion of ^{14}C-ImAA in both species after oral administration of gram amounts of Hi (Table IV) indicates that oxidative deamination plays a more important role in detoxication of large doses than of small doses It is worth mentioning that the increased formation of ^{14}C ImAA in goats was accompanied by a marked decrease in the fraction assumed to be splitting products of the imidazole ring of Hi The percentage increase of ^{14}C Hi metabolites in the urine was not however followed by any significant change in the amount of ^{14}CO excreted

The decrease in ^{14}C 1 4 MeHi relative to ^{14}C 1 4 MeImAA observed on oral loading in goats (Table I and IV) probably does not reflect an inhibition of the methylation pathway but rather an increased oxidative deamination of 1 4 MeHi Change in the metabolic pattern for Hi on oral loading may be partly explained by a dose dependent distribution of Hi within the gastrointestinal tract and between different tissues with varying activity of the Hi metabolizing enzymes Thus about 50 % of the given radioactivity was calculated to remain in the rumen 9 h after administration of 1 2 g Hi while less than 5 % was found at the same time when only ^{14}C Hi was given Oral loading of Hi may change the turnover rate in the rumen 1) By influencing the transport to the intestines 2 By changing the absorption of Hi and its metabolites and 3) By changing the catabolism of Hi which again can influence the absorption

In the cow it has been reported that 2—5 mg Hi given i v gave an immediate though temporary paralysis of the rumen muscles (Dougherty 1942 It seems likely that paralysis may occur after oral administration of gram doses in all ruminating species In experiments with sheep in which polyethylene glycol PEG a substance not absorbed from the intestine) and small amounts of ^{14}C Hi were given together

Sjaastad (1967) found a marked difference in the disappearance rate of radioactivity and PEG from the rumen. Sjaastad assumed that this difference might entirely be due to absorption of inactivation products of Hi since he had earlier found that Hi itself was absorbed from the rumen of sheep only to a very low extent. In the present study the disappearance rate of radioactivity from the rumen of the goat given 1.2 g Hi corresponds well during the first hours with that found by Sjaastad for PEG in sheep. This would indicate an inhibition of the Hi catabolism. A transient substrate inhibition of Hi catabolism was also indicated by a marked delay in the excretion peak of CO (Fig. 1—4). The radioactivity in the blood plasma after BaCl treatment showed that only a small part of the radioactivity in the plasma was due to the presence of ^{14}CO. High specific radioactivity of ^{14}CO expired at a time when the radioactivity in the rumen is low (Fig. 4) indicates therefore that ^{14}CO formed in the rumen is delayed in the tissue.

Effects of intravenous Hi loading on the metabolism of injected ^{14}C Hi

The fact that i.v. loading with milligram doses of Hi did not change the fraction recovered unchanged in the urine and that this treatment did not alter the metabolic patterns to any large extent in either pigs or goats indicates that the two examined species are well equipped to metabolize even large doses of Hi reaching the systemic circulation. However, toxic manifestations appeared within the first minute of intravenous loading with Hi. This was not unexpected since the dose given was relatively large and since it was given over a very short period of time. Thus the blood concentration of Hi must be very substantial before Hi is inactivated. Since the percentage unmetabolized Hi excreted with the urine was not changed by Hi loading the total amount of free Hi must increase. This was observed (Table I). In pigs the toxic effect of the given Hi may be potentiated by an apparent releasing effect as indicated by the fact that the increment of Hi metabolites exceeded the quantities of Hi given. In goats, however, such a great increment of unlabelled Hi metabolites was not observed, but it is worth mentioning that the main metabolite of i.v. injected Hi in goats — ImAA — was not determined since methods to determine nonlabelled ImAA originating from Hi were not available. As mentioned above the large increment of Hi metabolites in the urine of pigs must partly be due to liberation of Hi and/or Hi metabolites. Increased Hi formation due to Hi loading seems very unlikely. However, it should be mentioned that a substantial increase in the excretion of Hi metabolites was also observed during the second 24 hour period after loading (Table I, expt. no. 14) which would indicate that the apparent release of Hi was longlasting. The apparent releasing effect of Hi will have to be examined more closely also in other species.

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A Comparative Study of in vitro Metabolism of Histamine in Various Tissues from Domestic Animals (Cow, Sheep, Horse and Pig)

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Abstract

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Previously the *in vivo* metabolism of histamine in domestic animals has been examined Ruminating and non ruminating species seemed to metabolize both parenterally and orally administered Hi in different ways The present report is concerned with the ability of various tissues from the two types of animal to metabolize histamine In all species kidney was the most active and lung one of the least active histamine degrading tissues In ruminating species liver seemed to be the most active tissue next to kidney while intestinal mucosa was of relatively minor importance In the non ruminating species mucosa of the small intestine was the most active tissue after kidney while liver was less active Imidazoleacetic acid and an unidentified "metabolite XI were the 2 main metabolites of histamine in most tissues examined both at low and high substrate concentrations The formation of metabolite XI was prevented by 10^{-4} M aminoguanidine Addition of S adenosylmethionine to the incubation mixtures increased the inactivation rate of histamine in the lung of all species examined in the intestinal mucosa of the ruminants and in the liver of the non ruminants 1 4 MeH did not seem to be oxidized to any extent in the methylating tissue

From experiments with goats sheep horses and pigs in which the ^{14}C histamine metabolites in urine were examined after i v injection of ^{14}C histamine it seems likely that oxidative deamination is the major pathway for histamine degradation in ruminants while in non ruminating species methylation seems to dominate (Eliassen 1969 1971 a and b) The present report is concerned with the ability of various tissues from the two types of animal to metabolize histamine In most laboratory animals kidney and liver are two of the most active tissues in histamine degradation In kidney oxidative deamination is the main degradation pathway while in liver

Abbreviations Hi histamine 4(5) (β aminoethyl) imidazole AcHi N acetylhistamine 4(β acetylaminoethyl) imidazole 1 4 MeHi 1 4 methylhistamine 1 methyl 4 (β aminoethyl) imidazole ImAA imidazoleacetic acid imidazole 4(5) acetic acid 1 4 MeImAA 1 4 methylimidazoleacetic acid 1 methyl imidazole 4 acetic acid HiOH histaminol 4 imidazoyl ethanol

methylation often plays a certain role. Tissues from these two organs were therefore used in the present comparative study. Since high levels of histamine in the gastrointestinal tract have been suggested to be of importance in the pathogenesis of some diseases in domestic animals (Dougherty 1942, Dain, Neal and Dougherty 1955 and Akerblom 1939) and since in most common laboratory animals the mucosa of the small intestine has been found to be the most active tissue in the gastrointestinal tract in Hi degradation (Kim *et al.* 1969), it was also desirable to investigate the inactivating capacity of the tissue interposed between the digestive tract and the systematic circulation. In the present study duodenal and ileal mucosa was examined. Lung tissue was included in the present experiments because it is known to contain very high amounts of histamine.

Materials and Methods

Liver, kidney, lung, duodenal mucosa and ileal mucosa of adult female sheep of the Dala breed, of cows of the Norwegian red cattle breed (NRF), horses of Dala breed and female and castrated male pigs of the Norwegian Landsvin breed were obtained at a slaughter house. Data on the age of the animals were not available.

Pieces of liver, kidney, lung and segments of about 25 cm of duodenum and ileum were separately transferred to thermos flasks containing partly frozen Ringer phosphate solution (pH 7.4) as soon as possible after death. Usually this was accomplished within 10—20 min after slaughter. The segments of the intestines were cleaned with ice cooled Ringer solution before they were transferred to the thermos flasks. The segments of duodenum and ileum were taken about 25 cm behind the entrance of the bile duct and about 25 cm in front of the the ileo-caecal junction respectively. Usually different tissues from the same animal were incubated simultanously. In the experiments where S-adenosylmethionine was added, however, the same tissue from different animals was incubated simultaneously.

Preparation of tissue

During the preparation of the tissues the temperature was kept near 0° C. All tissue slices and pieces were gently blotted on filter paper just before aliquots (0.20—1.0 g) were weighed in ice cooled Ringer phosphate solution. The interval between slaughtering and starting the incubation never exceeded 2 1/2 h.

Kidney and liver. Slices with an average thickness of 0.1—0.3 mm of liver and kidney (cortex and medulla) were used. These were prepared using a tissue slicer (The Harvard Apparatus Co. Inc., Dover, Massachusetts, U.S.A.). Aliquots of 200 mg were used in incubation.

Lung. The pleura was removed and the lung was cut into small pieces using scissors. 1 g of tissue was then homogenized for 15 s in 4 ml of Ringer phosphate solution with an Ultra Turrax® homogenizer (Janke & Kunkel K. G., W. Berlin, Germany).

Duodenal and ileal mucosa. Peeling of the mucosa from the rest of the intestinal wall (muscle) was easily accomplished by means of curved tweezers. No histological examination were carried out, but the mucosa certainly contained some connective tissue in addition to the mucosa cells. Pieces of mucosa weighing 0.50—1.00 g were homogenized for 5—10 s in 4 ml of Ringer solution with an Ultra Turrax® homogenizer. Homogenates were used instead of intact tissue because it has been found for sheep that homogenate of small intestine mucosa are more effective in Hi degradation than the intact tissue (Sjaastad 1967).

Determination of tissue histamine

The Hi concentration of the tissues were determined in order to ascertain the total amount of Hi initially present in incubated tissue. One g tissue was used for all Hi determinations. The tissues were prepared in the same way as for incubation. The tissue preparation instead of being incubated was rapidly heated to boiling point in a water bath. After cooling 100 µl of N HCl was added making the pH below 2. The slices of kidney and liver were finally homogenized. All the homogenates were stored in a refrigerator at 4° C up to 1 day before assay without loss of biological activity. All tissue homogenates were neutralized with N NaOH and the homogenates of lung and of intestinal mucosa were diluted up to 50 ml with Tyrode solution before the Hi content was assayed on isolated atropinized (100 µg atropine sulfate/l)

guinea pig ileum The Hi like nature of the substance causing contractions was verified by antagonizing with the antihistamine Allergin® (Diphenhydramine chloride)

Incubation 25 ml Erlenmeyer flasks were used as incubation vessels Each flask contained 4 0 ml Ringer phosphate solution pH 7 4 The tissues were weighed directly into the vessel and the lung and intestinal mucosa tissue were homogenized in the vessels ^{14}C-Hi (5—8 µCi) and carrier Hi (12—650 µg) each dissolved in 100 µl Ringer phosphate solution were added thus the final volume excluding the volume of the tissues was 4 2 ml It has been shown that in homogenates of liver and kidney methylation of Hi is negligible unless S-adenosylmethionine is added (Lindahl 1958 and Kim 1959) Therefore some incubations were also done in the presence of this cofactor S adenosylmethionine added to the incubation mixtures was dissolved in 100 µl Ringer phosphate solution

The incubation took place in a water bath at 37 C and the incubation mixtures were shaken mechanically by moving the vessels 5 cm back and forth about 50 times per min After 2—2 1/2 h incubation was terminated by rapidly heating the incubation mixture to boiling point followed by addition of 100 µl 6 N HCl The kidney and liver slices were after heating homogenized with an Ultra Turrax® for 10 s The incubation mixtures were then centrifuged at 5000 × g for 10 min and the supernatant was frozen at −20 C and stored at this temperature until analysis Usually the pH of the incubation mixtures was checked after incubation and was found to be about 6 8

The incubations were not carried out under sterile conditions and antibiotics were not added The first because of practical difficulties and the second because it was feared that antibiotics could interfere with the Hi catabolism In a few instances the bacterial content in the incubation mixtures was estimated by plating the incubation mixtures on agar The highest total bacterial content about 60 000 was found in the incubation mixtures of the intestinal mucosa In the kidney and liver incubates never more than about 20 000 bacteria were found

Paper chromatography 50—100 µl of the supernatant of the incubation mixtures was chromatographed on paper for separation of histamine and its metabolites Whatman paper no 1 and the following solvent system were used t butanol formic acid 95 % pyridine (70 15 15 V V V) (Lindahl 1958)

To examine the identity of the compound designated "metabolite XI" two-dimensional paper chromatography as previously described was used (Eliassen 1969)

Together with the supernatant of the incubation mixture 10 µl of standard mixture containing Hi 2 HCl AcHi 1 4 MeHi HCl 1 4 MeImAA HCl and ImAA HCl (5 µg/µl) was applied to the paper The radioactive metabolites were identified by radioautography and by visualization of the carrier substances on the chromatograms by spraying the chromatograms with one or both of the following two reagents 1) diazotized p-nitroaniline followed by 10 (w/v) Na_2CO_3 and 2) 1 (w/v) iodine in ethanol

Determination of Hi metabolites and estimation of the Hi inactivation rate

Semiquantitation of ^{14}C Hi and its metabolites was obtained by counting the radioactive areas on the paper chromatograms in a Beckman Lo beta II flowcounter (background 1 8—2 8 cpm) By measuring the decrease of ^{14}C H in the incubation mixtures the percentage inactivation of Hi could be calculated Since the initial concentration of non labelled Hi in the incubation mixtures was known also the inactivation rate for Hi expressed as µg per g wet weight of tissue per hour could be calculated Since the H inactivation was determined by difference low accuracy would be obtained in case of low percentage inactivation Thus an inactivation of 1—2 % would be determined with a coefficient of variation of about 54 the corresponding figures for 7—11 23—52 % and 54—91 % inactivation would be about 16 6 and 4 respectively By adding Hi in steps of 12—50 µg and 35—150 µg to tissues with low and high inactivation capacity respectively and by measuring the Hi inactivation at each concentration the optimal substrate concentration could be roughly estimated The metabolic pattern of Hi at the substrate concentration which prevailed when no Hi was added and at about optimal substrate concentration for H inactivation was determined as indicated above by means of paper chromatography All H values refer to Hi base

Materials

Histamine (2 ring ^{14}C spec act 54 mCi/mmol was obtained from the Radiochemical Centre Amersham England 1 4 MeH and 1 4 MeImAA were synthesized after a procedure used at Clin Physiological Laboratory Lasarettet Lund Sweden (Head H Westling) Histamine dihydrochloride and diphosphate imidazoleacetic acid hydrochloride S adenosylmethionine chloride and p-hydroxymercuribenzoate were purchased from Sigma Chemical Co St Louis U S A N acetylhistamine was purchased from Calbiochem Los Angeles U S A Aminoguanidine sulfate was purchased from Eastman Organic Chemical Rochester 3 N Y U S A Allergin® (Dyphenhydramine chloride) was purchased from Nyegaard & C[illegible] Oslo Norway

Results

Concentration of Hi in the tissues

The initial concentration of free Hi in the tissue prior to the incubation is given in Table I There was a great difference in the Hi content between different tissues and the Hi content in the same tissue could vary greatly within the same species The Hi concentration in liver and especially in kidney was very low in all examined species With the exception of the sheep the highest Hi content was found in the intestinal mucosa about 10 times that found in liver In the ruminating species there was a tendency to a higher Hi content in the duodenal mucosa than in the ileal mucosa A relatively high content in one tissue did not indicate correspondingly high values for the other tissues in the same animal

TABLE I Histamine content in different tissues of cows sheep horses and pigs Single observations or mean ± S D (n)

Organ	Cow µg Hi/g wet weight		Sheep µg Hi/g wet weight	Horse µg Hi/g wet weight	Pig µg Hi/g wet weight	
Liver	5 6±3 6	(4)	0 7–3 6	3 3±2 1 (5)	6 3±3 4	(9)
Kidney	1 0±0 7	(4)	0 04–0 06	1 0–3 5–3 6	0 9±0 3	(8)
Lung	37±18	(6)	11–78	17±6 (4)	21±18	(5)
Duodenal mucosa	60±15	(7)	18±7 (4)	24±9 (4)	68±10	(7)
Ileal mucosa	49±10	(4)	10–11	20–21 –43	69±24	(5)

Determination of the substrate concentration for maximal Hi inactivation

The results from experiments without addition of S-adenosylmethionine are given in Table II Since the inactivation was determined by difference as indicated above low accuracy was obtained in the case of low percentage inactivation For cows the optimal substrate concentration for Hi degradation seemed to be of the same order for all tissues tested The optimal substrate concentrations for Hi degradation in

TABLE II Metabolism of histamine *in vitro* Approximately optimal substrate concentration and the corresponding inactivation rate Single observations or mean ± S D (n)

Species	Liver				Kidney			
	Substrate µg		Inactiv rate µg g h		Substrate µg		Inactiv rate µg g/h	
Cow	75 –	145	103 62	(5)	75 –	145	154±74	(5)
Sheep	100	100	43 37		75	222	254 134	
Horse	18	5	1 11 18		145 –	360	210 400 580	
Pig	1 –	30	6 5 6 0	(4)	270 –	360	473±52	(4)

TABLE II continuation

Lung		Duodenal mucosa		Ileal mucosa	
Substrate µg	Inactiv rate µg/g/h	Substrate µg	Inactiv rate µg/g/h	Substrate µg	Inactiv rate µg/g/h
10 — 110	18±7 (5)	55 — 110	26±11 (5)	55 — 160	19±9 (5)
36 90	2 5	90 160	7 4	160 310	5 9
50 — ?00	1 4 5	55 — 160	36 40 61	160 310	116 180
4 — 7?	3 0±1 8 (4)	400 — 490	133±32 (5)	380 — 670	144±2? (5)

kidney lung and duodenal mucosa of sheep were about the same as for cows In horses and pigs the lowest values for the optimal substrate concentration were found for the liver and lung tissue On the other hand the values for kidney and small intestinal mucosa especially in pigs were very high The optimal substrate concentration for H_1 inactivation in lung tissue was with the exception of cows usually identical with the preformed amount of H_1 Furthermore as for the intestines there was with the exception of cows a tendency to higher values for the optimal substrate concentration in ileal mucosa than in duodenal mucosa In tissues containing high and variable concentrations of H_1 combined with a low inactivation capacity for H_1 for example lung tissue there will be a very high degree of inaccuracy in the estimation of the substrate concentration giving maximal inactivation rate

If the substrate concentration in the liver and kidney mixtures was increased above the optimal substrate concentration for the H_1 degradation a marked inhibition of the H_1 inactivation was observed Such an inhibition was often observed also in the intestinal mucosa but it was not so pronounced in this tissue

The H_1 inactivation rate in the tissues at about optimal substrate concentration The results are given in Table II Kidney tissue seemed in all species to have the highest and lung tissue the lowest capacity to inactivate H_1 In the ruminating species liver tissues seemed to be most active in H_1 inactivation next to the kidney tissue while the intestinal mucosa had a very low H_1 inactivating capacity On the other hand in the non ruminating species the intestinal mucosa had a high while liver tissue had a very low capacity to inactivate H_1

The metabolic pattern for ^{14}C H_1 in different tissues
The results show great variations in the metabolic pattern of H_1 from one time to another but within the great variations there seemed to be some significant differences between species and between organs (Fig 1a—1d)

2—13 % of the radioactivity was located at the application point on the paper chromatograms and was probably not due to specific H_1 metabolites In the chromatographic system used AcH_1 and H_1OH did not separate The radioacti-

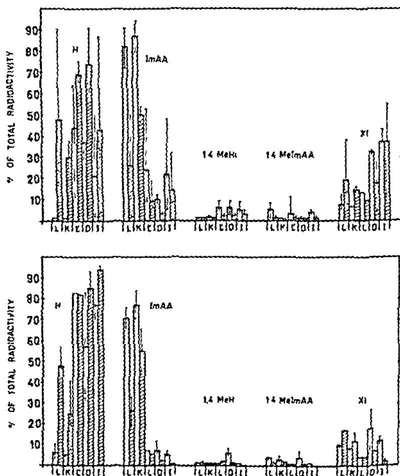


Fig. 1 a–b. The main ^{14}C Hi metabolites in different tissues of a) cows and b) sheep after incubation for 3 1/2 h without carrier Hi (dotted bars) and with carrier Hi added to about optimal inactivation rate (cross-hatched bars). The bars represent the mean and the vertical lines the range of 4 and 2 expts. for cows and sheep, respectively. The tissues from left to right: L = liver, K = kidney, L = lung, D = duodenal mucosa and I = ileal mucosa

associated with the location of these two metabolites was very low and often insignificant for all tissues and animals. In most of the chromatograms a radioactive compound with Rf of about 0.17 gave a significant spot on the X-ray films (Fig. 2). Judged from two-dimensional paper-chromatography the unidentified compound designated metabolite XI in the present study is not identical with ImAA riboside. Systematic experiments in order to identify this compound have up to the present time not been made.

As earlier mentioned, kidney was in all species the most effective tissue in Hi metabolism. In this tissue oxidative deamination of Hi to ImAA was quantitatively the most important degradation pathway of Hi, independent of substrate concentration. Next to ImAA, metabolite XI was the main Hi metabolite, especially at high substrate concentrations.

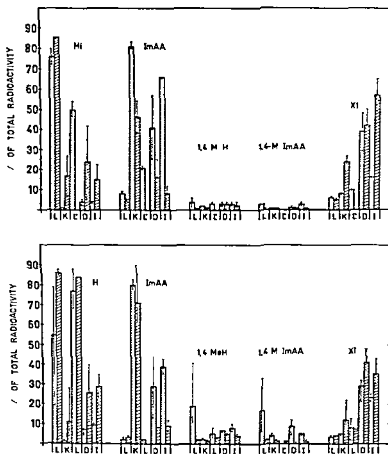


Fig 1 c—d The main ^{14}C-Hi metabolites in different tissues of c) horses and d) pigs after incubation for 2 1/2 h without carrier Hi (dotted bars) and with carrier Hi added to about optimal inactivation rate (cross-hatched bars) The bars represent the mean and the vertical lines the range of 3 and 2 expt for pigs and horses respectively The tissues from left to right L = liver K = kidney L = lung D = duodenal mucosa and I = ileal mucosa.

In lung the tissue found to have the lowest Hi inactivation capacity the main Hi metabolites were metabolite XI and ImAA In lung of pigs methylation seemed also to play a relatively significant role

In the ruminating species liver tissue seemed effective in Hi inactivation At low Hi concentration ImAA appeared in the highest quantity However at about optimal substrate concentration metabolite XI and ImAA occurred in similar amounts In the liver tissues of horses and pigs only a small part of Hi was inactivated At low substrate concentrations the methylated compounds accounted in the pig liver tissue for the greater part of the radioactivity while in horse liver both ImAA and metabolite XI were of greater importance than the methylated metabolites

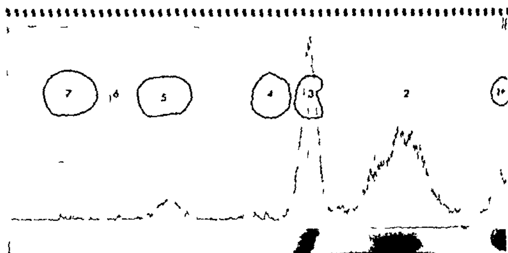


Fig. 2. An autoradiogram and a scanning diagram of a paperchromatogram on horse duodenal mucosa homogenates incubated with ^{14}C Hi and 150 μg Hi carrier for 3 1/2 h. The substances indicated are as follows: 1. Application point, metabolite XI, 3. Hi, 4. 1,4-MeHi, 5. ImAA, 6. 1,4-MeImAA, 7. AcHi and HiOH. Spots are those which were made visible by diazotized p-nitroaniline and Na_2CO_3. The scanning diagram was made with the help of a Packard Radiochromatogram Scanner. Speed 1 cm/min. Max deflection 1 000 cpm.

No marked difference between duodenal and ileal mucosa regarding the metabolic pattern for Hi could be seen in any of the species. ImAA and metabolite XI were in the intestinal mucosa of all species the 2 main Hi metabolites. In the ruminants metabolite XI tended to account for a relatively greater part of the radioactivity than ImAA independent of the substrate concentration. At high substrate concentration this was also the case in horses and pigs.

When homogenized duodenal mucosa of pig (with 400 μg Hi2P added) was incubated in the presence of 10^{-4} M aminoguanidine, the Hi inactivation including the formation of metabolite XI was almost completely abolished. When 10^{-3} M p-hydroxymercuribenzoate was used instead of aminoguanidine, no change in the Hi metabolism could be observed.

Effect of S-adenosylmethionine on the in vitro Hi metabolism

Preliminary experiments showed that S-adenosylmethionine had only a very small or no effect on Hi metabolism in the following tissues: kidney of all species, liver of the ruminants and intestinal mucosa of horses and pigs. However, the Hi inactivation rate in duodenal mucosa of both sheep and cows was increased by addition of S-adenosylmethionine to the incubation medium, but only in cows did the optimal substrate concentrations increase significantly (Table III).

Preliminary experiments showed that the effect of S-adenosylmethionine was similar in ileal and duodenal mucosa. In liver slices, especially of pigs but also of horses, addition of S-adenosylmethionine increased the Hi inactivation rate. The optimal substrate concentration for Hi inactivation in liver increased significantly

TABLE III Metabolism of histamine *in vitro* Effects of S adenosylmethionine on optimal substrate concentration and inactivation rate at approximately optimal substrate concentration

Tissue	Species	S adenosylmethionine μmol	Approximately optimal substrate conc μg			Hi inactivation rate μg/g/h		
Duodenal mucosa homogenates	Cow	0	35	56	54	13	19	20
		1 0	145	206	204	24	26	25
Duodenal mucosa homogenates	Sheep	0	111	124		12	6	
		1 0	111	124		18	15	
Liver slices	Horse	0	32	25	50	8	6	14
		0 5	32	25	50	9	11	17
Liver slices	Pig	0	14	13	25	4	4	8
		0 5	51	51	50	31	35	29
Lung homogenates	Cow	0	21	28		3	4	
		1 0	96	228		24	64	
Lung homogenates	Horse	0	9			1		
		1 0	34			12		
Lung homogenates	Pig	0	38	68		4	6	
		1 0	138	143		2[illegible]	29	

only in pigs (Table III) Addition of S adenosylmethionine to lung homogenates caused marked increase of both the inactivation rate and the optimal substrate concentration in all species examined The increased Hi inactivation rate which occurred when S adenosylmethionine was added was mainly due to an increased methylation of Hi to 1 4 MeHi No apparent oxidation of 1 4 MeHi to 1 4 MeImAA occurred to any extent in the methylating tissue A significant increase in the formation of 1 4 MeImAA could only be seen in the liver of pigs and in the liver and lung preparations of horses (Fig 3 a—b) The increased formation of 1 4 MeHi seemed especially in duodenal mucosa to be followed by a decrease in ImAA and metabolite XI

Discussion

The present study shows that the differences between ruminants and non ruminants in the metabolism of Hi are demonstrable not only *in vivo* but also using *in vitro* technique However the observed differences in the metabolic pattern of Hi in the examined tissues can not alone explain the *in vivo* differences between the two types of animals especially regarding the ability to methylate Hi Thus *in vivo* methylation is the main metabolic pathway for Hi degradation in horses and pigs (Eliasen 1971 b) but taken as a whole the examined tissues from these species mainly detoxicate Hi by oxidative deamination Mouse is also a species which mainly methylates parenterally administrated Hi (Schayer 1956) In liver slices of this species methylation of Hi is highly predominant (Lindahl 1958) However in homogenates of mouse liver addition of S adenosylmethionine was necessary to obtain methylation (Lindahl 1958) The possibility exists that the low degree of

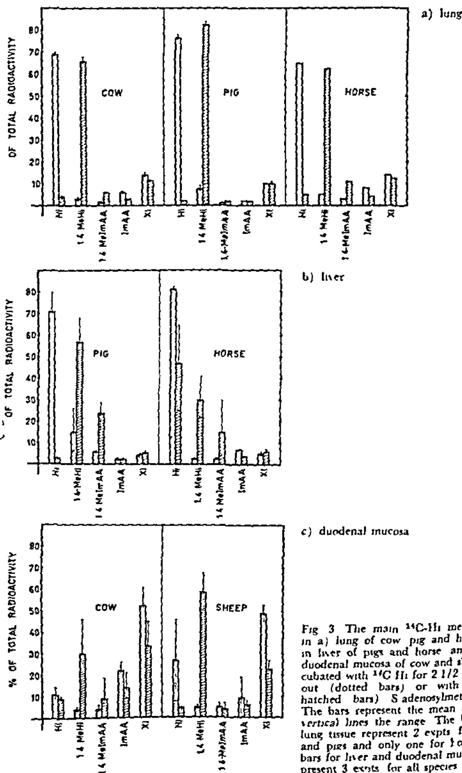


Fig 3 The main ^{14}C-Hi metabolites in a) lung of cow pig and horse b) in liver of pigs and horse and c) in duodenal mucosa of cow and sheep incubated with ^{14}C Hi for 2 1/2 h without (dotted bars) or with (cross-hatched bars) S adenosylmethionine The bars represent the mean and the vertical lines the range The bars for lung tissue represent 2 expts for cows and pigs and only one for horse the bars for liver and duodenal mucosa represent 3 expts for all species

methylation which was observed in all tissues in the present study also could be due to a shortage of coenzymes This seemed to be true for some tissues since addition of S adenosylmethionine to the incubation mixtures resulted in a markedly increased methylation of Hi in lung homogenates of both types of animals in the liver slices of pigs and horses and in the intestinal mucosa homogenates of the ruminants (Table III and Fig 3 a—c) The inactivation rate in the intestinal mucosa of ruminants was however even after addition of S adenosylmethionine relatively low compared with the intestinal mucosa of the non ruminating species the liver of the ruminants and the kidney of all examined species Under the assumption that *in vitro* experiments to some extent reflect what happens *in vivo* the present study indicates that the examined tissues are only to a small extent the source of urinary methylated Hi metabolites Other tissues than those examined must be the main sources However it should be mentioned that the findings of Granerus Wetterqvist and White (1968) indicate that Hi injected or infused into the blood stream is methylated less efficiently than Hi released within the tissues The increased formation of 1 4 MeHi without any special increase in 1 4 MeImAA in some tissues to which S adenosylmethionine was added indicates that the oxidative deamination of 1 4 MeHi probably does not occur in those tissues in which it is formed

Experiments with i v Hi loading have previously shown that goats and pigs are well equipped to metabolize large amounts of Hi (Eliassen in press) The high Hi inactivation capacity found in kidney tissue in the present experiments indicates that the kidney in the examined species must be very important in the detoxication process of Hi reaching the systemic circulation Even assuming a sufficient supply of S adenosylmethionine *in vivo* liver in pig and horses seemed compared with kidney to have little quantitative importance in the detoxication of Hi The capacity of the lung to inactivate Hi is also relatively low even after addition of S adenosylmethionine The inactivation capacity of the lung tissue is however probably mainly of importance for detoxication of Hi liberated from the lung tissue itself The fact that liver and small intestine mucosa preparations from the ruminants and the non ruminants mainly inactivate Hi at different rates and in different ways after addition of S adenosylmethionine seemed to be of special interest since these two types of animals have quite different digestive processes The high Hi inactivation rate of the small intestine mucosa of horses and pigs agrees well with that found in most other species (Waton 1956) If as would seem to be indicated from the considerable formation of ^{14}CO after oral administration of ^{14}C Hi (Eliassen in press) ingested Hi is inactivated within the fore stomach in the ruminants by microorganisms a high inactivation capacity in the small intestinal tissue does not seem necessary However in ruminants liver possesses a large reserve capacity for the inactivation of Hi entering the portal system On the other hand in horses and pigs where all small intestine mucosa is well equipped to metabolize Hi absorbed from the digestive tract liver seems to be of less importance in this respect

The inactivation capacity and the substrate concentration giving maximal inactivation in small intestine mucosa liver and kidney tissues of sheep correspond

well with findings in previous experiments with sheep using biological methods for estimation of Hi (Sjaastad 1967 a b and c) The Hi inactivation rate for liver and kidney of cows and pigs also agrees well with previous findings (Holtz Heise and Spreyer 1938) As found by Sjaastad (1967 a b and c) for sheep tissues a large decrease in the Hi inactivation rate was observed in the examined tissue of all species especially in the liver and the kidney tissues when the substrate was increased above a certain concentration This indicates a substrate inhibition The *in vitro* inhibition of Hi inactivation at high substrate levels corresponds well with results of *in vivo* experiments with rats (Schayer Kennedy and Smiley 1953) in which the degradation of Hi was roughly proportional to the dose until a maximum was reached after which the rate of Hi degradation decreased

In previous experiments (Eliassen 1971 a and b and in press) conjugated ^{14}C Hi had been found in urine after oral administration of ^{14}C Hi but not after intravenous administration The present study supports the conclusion from these experiments that conjugation of Hi takes place in the lumen of the gastrointestinal tract and probably not in the tissue Metabolite XI which was found especially in the incubation mixtures of small intestine mucosa in horses pigs and cows has not been identified Diamine oxidase (E C 1 4 3 6) may possibly participate in its formation since 10^{-4} M aminoguanidine prevented its formation

The great variation in the inactivation rate which was observed between different animals within the same tissue and between the different tissues also seemed to exist for the Hi content of the tissues (Table 1) However there was no correlation between the Hi content of the tissue and the Hi inactivation rate Thus tissue with high Hi inactivation rate could contain either large or small amounts of Hi This was the case for intestinal mucosa and kidney of pigs respectively Some of the differences in tissue Hi content between animals may have arisen after death even though care was taken in handling the tissues identically in all experiments However the tissues Hi values correspond well with those previously found in sheep (Sjaastad 1967 a b and c) horses and cows (Feldberg 1956)

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Effects of Chlorpromazine, Imipramine, and Quinidine on Action Potential and Tension Development in Single Skeletal Muscle Fibres of the Frog

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Abstract

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The effects of chlorpromazine, imipramine and quinidine on resting membrane potential, action potential, twitch and tetanic tensions, and on the active state were investigated in single frog skeletal muscle fibres. Chlorpromazine in concentrations 10^{-6}—10^{-5} M and imipramine and quinidine in concentrations 10^{-5}—5×10^{-5} M did not affect the resting membrane potential but produced a dose-dependent decrease in the rate of rise, rate of fall and overshoot of the action potential. The duration measured at -50 mV was prolonged. The drugs potentiated the isometric twitch by increasing the duration of the active state. Tetanic tension was not affected. In high concentrations (chlorpromazine $\geqslant 3 \times 10^{-5}$ M, imipramine and quinidine $> 5 \times 10^{-5}$ M) the drugs caused a successive widening and decrease of amplitude of the action potential and finally blocked the electrical activity. After a marked initial increase, the peak twitch tension was reduced in parallel with the changes of the action potential. The reduction in twitch amplitude was probably due to incomplete activation of the fibres. The mechanisms of the effects on the excitation-contraction coupling induced by chlorpromazine, imipramine and quinidine are discussed.

The processes linking the reactions of the plasma membrane during excitation to the activation of the contractile system in a muscle can be altered by drugs (for review, see Sandow 1965). Chlorpromazine, imipramine and quinidine have effects which may influence more than one of the steps involved in the excitation—contraction coupling. These drugs have been shown to reduce the active uptake of calcium in isolated sarcoplasmic reticulum (Balzer, Makinose and Hasselbach 1968, Fuchs, Gertz and Briggs 1968, Worsfold and Peter 1970). They also lower the mechanical threshold and prolong the relaxation phase of potassium induced contractures in single skeletal muscle fibres (Andersson 1972 a). Furthermore, these drugs are membrane stabilizers (Shanes 1958 a, b, Seeman 1966, Langslet 1970, Langslet *et al.* 1971) and inhibit the passage of calcium and other ions across cell membranes (van

Zwieten 1969 Balzer and Hellenbrecht 1969) It has been suggested that because of these effects the drugs would be able to inhibit the release of calcium from the sarcoplasmic reticulum and thus diminish the contractile response to direct stimulation of the muscle (Balzer and Hellenbrecht 1969)

Reports on the effects of chlorpromazine and imipramine on tension development of electrically stimulated skeletal muscle are sparse However in frog sartorius and rectus abdominis muscles chlorpromazine was shown to diminish the twitch amplitude (Balzer and Hellenbrecht 1969) Quinidine and its optical isomer quinine on the other hand have been more extensively investigated and are known to potentiate the twitch response (Harvey 1939 Krayer and George 1951 Isaacson Yamaji and Sandow 1970) by prolonging the duration of the active state (Lammers and Ritchie 1955) Reports of a reduced tension development in the twitch induced by quinidine have also appeared (Balzer and Hellenbrecht 1969)

Most previous studies of the effects of chlorpromazine imipramine and quinidine on the mechanical activity of skeletal muscle have been performed on whole muscle When the effects of these drugs on the cellular level are investigated a single fibre preparation is preferable In the present study therefore the effects of chlorpromazine imipramine and quinidine on resting membrane potential action potential and tension were investigated in single skeletal muscle fibres of the frog

Methods

Preparation and mounting Single muscle fibres were dissected from the ventral head of the semitendinosus muscle of *Rana temporaria* Frogs from all seasons of the year were used The fibres were mounted horizontally in a jacketed Perspex chamber designed for rapid exchange of solutions as previously reported in detail (Andersson 1972 a b)

Recording The techniques for determinations of sarcomere length at rest and for recording of isometric tension and active state were essentially the same as described previously (Edman 1966 Edman and Kiessling 1971) Resting membrane potential and action potentials were recorded intracellularly by means of conventional glass capillary electrodes The first derivative of the action potential was obtained by electrical differentiation using an RC-circuit with a time constant of 0 1 ms The experiments were performed at sarcomere lengths 2 3—2 5 μm

Stimulation The fibres were stimulated by passing current through a multielectrode assembly consisting of 5 platinum wires (0 125 mm diameter) spaced at 2 mm intervals along the length of the fibre The wires were arranged as alternate anodes and cathodes The stimulus strength was adjusted to ensure that each pair of electrodes produced supramaximum stimulation Square pulses of 0 9 ms duration were used Tetanic contractions were produced by application of a 1 s train of pulses of a frequency of 30—40 Hz The fibres were mounted 1—3 h before the experiment and were tetanized occasionally during that time Only fibres producing completely fused tetani were used Multi-electrode stimulation was used in experiments where the mechanical activity (twitch tetanus active state) was recorded For action potential recordings the fibre was locally stimulated through a single pair of platinum wire electrodes The fibre was stimulated at one end and the microelectrode inserted in the middle part or at the opposite end of the fibre

Solutions The normal Ringer's solution had the following composition (mM) NaCl 115 5 KCl 2 0 CaCl 1 8 NaH_2PO —Na_2HPO 2 0 pH 7 0

In some experiments a hypertonic solution was used to mechanically inactivate the fibres This solution was prepared by adding urea (final concentration 400 mM) to the normal Ringer's solution

All solutions were prepared on the day of the experiments Double distilled water and analytical grade chemicals were used

Temperature. The temperature of the bathing solution was controlled by circulating a water ethylene glycol mixture through the jacket around the chamber by means of a Calora Ultra thermostat. During any given experiment the temperature of the bath was constant to ±0.1 C and varied from 6.5 to 7.5 C between different experiments.

Drugs. The following drugs were used: Chlorpromazine chloride (Hibernal, Leo AB), imipramine hydrochloride (Tofranil, Geigy), quinidine sulphate (Sigma Chemical Company).

Experimental procedure

a. *Recording of action potential.* Action potentials were recorded in the presence of chlorpromazine (10^{-6}—3×10^{-4} M), imipramine and quinidine (10^{-}—10^{-4} M). Recordings were made from 1) isolated single fibres. In these experiments the electrical and mechanical activities were recorded at the same time. The fibres were given single shocks with intervals of 1 or 2 min during 10—20 min. When reproducible twitches were obtained an impalement was made and the action potential and the twitch were recorded simultaneously. The same procedure was followed in both normal Ringer's solution and after addition of the drugs to be studied. During these experiments the fibres were often damaged as the microelectrode tended to tear the sarcolemma during the mechanical activity. Only the fibres that maintained their resting membrane potential within more than 9[illegible] per cent of the original value and produced twitches of unchanged amplitude after the first impalement were used for further experiments. In most fibres it was only possible to test one concentration of drug, but in a few the effects of ? concentrations could be studied and also the reversibility of the effects after returning the fibre to normal Ringer's. 2) Bundles of muscle fibres (3—6 fibres in each bundle). Bundles with fibres of approximately the same diameter were selected. The bundles were stimulated with the same time intervals as described above for single fibres. As the differences in action potential characteristic between the fibres within a bundle were found to be insignificant, 1 [illegible] fibres were taken as a control and action potentials were recorded in normal Ringer's solution. In the remaining fibres action potentials were recorded in the presence of one of the drugs to be studied. Some bundles provided action potential data only from normal Ringer's or from one of the drug-containing solutions. 3) Isolated single fibres mechanically inactivated by immersion in hypertonic urea Ringer. Action potentials were recorded before and 15—?0 min after addition of chlorpromazine, imipramine or quinidine to the hypertonic solution.

b. *Recording of twitch and tetanus.* When immersed in normal Ringer's solution the fibres were first stimulated with single shock at intervals of 1 or [illegible] min for 10—?0 min. When the twitches obtained during this period were reproducible, the twitch tension amplitude was used as a control value. The fibres were then stimulated tetanically (frequency 30—40 Hz, duration [illegible]) with interval of 3 [illegible] min. 4 or 5 tetani were produced. The mean of the tension values obtained during the last ? tetani was taken as a control. The solution of the chamber was changed to Ringer containing chlorpromazine, imipramine and quinidine in the same concentrations as in the action potential experiment. After a new control period with unchanged stimulation frequency twitches were recorded during 1—2 h. During this time the tetanus tension was also controlled.

c. *Recording of active state.* For determination of the decay phase of the active state the modification of Ritchie (1954) quick release method described by Edman (19[illegible]0) was used. This method also provides an approach for analysis of the rising phase of the active state. In a few fibres the duration of the maximum activation of the active state during a single twitch was determined according to the method of Macpherson and Wilkie (1954).

Results

Drug effects on resting membrane potential and action potential

a. *In normal Ringer's solution.* The resting membrane potential was not appreciably changed by the drugs in the concentrations used. In 9 fibres immersed in Ringer's containing chlorpromazine [illegible] 10 M (3 fibres), imipramine 10^{-4} M (3 fibres) and quinidine 10^{-4} M (3 fibres) the resting membrane potential was measured by impalements every 30 minutes during 5 h. In no fibre did the membrane potential values vary by more than ±3 mV during this observation period.

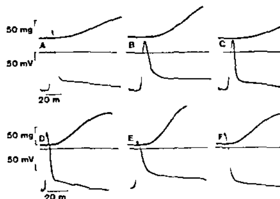


Fig 1 Action potentials and twitch tensions recorded before during and after exposure to quinidine and imipramine in 2 single fibres Upper traces A—normal Ringer's B—quinidine 5×10^{-5} M in normal Ringer's C—17 min after return to normal Ringer's Lower traces D—normal Ringer's E—imipramine 5×10^{-5} M in normal Ringer's F—18 min after return to normal Ringer's

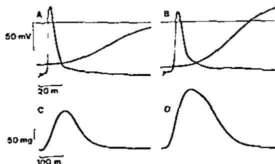


Fig 2 Action potentials and twitch tensions in a single fibre recorded in normal Ringer's (A and C) and in the presence of chlorpromazine 10 [illegible] M (B and D) The twitches in panels C and D were recorded immediately before the action potentials

TABLE I Effects on resting membrane potential (RMP) and action potential (AP) by different concentrations of chlorpromazine, imipramine and quinidine (mean and S E)

Solution	Number of bundle	Total number of fibres impaled	RMP mV	Overshoot mV	Maximum rate of rise of AP V	Maximum rate of fall of AP V	Duration of AP at −50 mV ms
Ringer's	18	26	91.5±0.4	40.3±0.7	152.5±3.4	41.7±1.2	4.6±0.07
Chlorpromazine 5×10^{-6} M	5	18	91.3±0.5	32.0±0.9	125.6±2.1	32.0±0.3	5.4±0.07
Chlorpromazine 10^{-} M	6	19	92.0±0.4	26.9±0.8	101.4±2.9	24.4±0.7	6.4±0.10
Imipramine 10 M	6	20	91.1±0.4	31.7±1.6	114.7±3.3	29.1±0.8	5.6±0.16
Imipramine $5\times10^{-}$ M	6	21	92.0±0.6	22.0±1.9	81.2±4.[illegible]	16.1±0.9	8.9±0.44
Quinidine 10 M	6	24	91.8±0.6	32.1±0.5	114.2±2.2	33.9±0.5	5.2±0.06
Quinidine $5\times10^{-}$ M	5	18	92.8±0.7	19.9±1.0	81.7±3.4	19.6±0.1	7.2±0.[illegible]

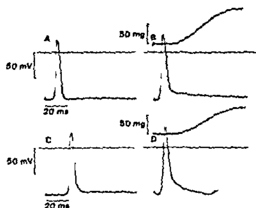


Fig. 3. Action potentials recorded in 2 different single fibres in urea Ringer's (A and C). Note absence of afterpotentials. Panel B and D show recordings after return to normal Ringer's solution.

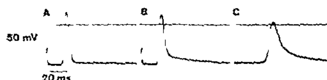


Fig. 4. Action potentials of a single fibre in urea Ringer's without (A) and with imipramine in concentrations 10^{-5} M (B) and 5×10^{-5} M (C).

As can be seen in Fig. 1 and 2, chlorpromazine, imipramine and quinidine had similar effects on the action potential: the rate of rise, rate of fall and overshoot were all reduced whereas the duration measured at -50 mV was increased. The degree of change induced by a given drug concentration varied from fibre to fibre but the effects were dose dependent (Table I) and to a great extent reversible in the fibres exposed to the lower concentrations of the drugs. With high drug concentrations (chlorpromazine $\geq 3\times10^{-5}$ M, imipramine and quinidine $>5\times10^{-5}$ M) and long exposure times irreversible changes were produced. A successive widening and decrease of amplitude of the action potential was observed before the electrical activity was blocked. In parallel with these action potential changes there was a reduction of the mechanical response.

b. *In urea Ringer's solution.* In the fibres immersed in urea Ringer's the mechanical activity usually disappeared within one minute and did not return during the 10—20 min of immersion. After returning the fibre to normal Ringer's solution the mechanical activity recovered rapidly and after a short period (5—10 min) during which a slight potentiation could be seen, the twitch reached the control value. Urea Ringer's did not appreciably change the resting membrane potential in most fibres (a few depolarized by 4—8 mV) and also the action potential was little affected. In six out of eleven fibres, however, a completely reversible decrease or disappearance of the negative afterpotential (Frank 1957) could be demonstrated in urea Ringer's (Fig. 3). Also in these fibres the effects on the mechanical activity were completely reversible. The drug induced changes of the action potential seen

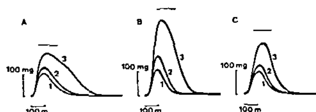


Fig 5 Twitch tensions of three different single fibres (A B and C) exposed to A normal Ringer's (1) chlorpromazine 5×10^{-6} M (2) chlorpromazine 10^{-5} M (3) B normal Ringer's (1) imipramine 10^{-5} M (2) imipramine 5×10^{-5} M (3) C normal Ringer's (1) quinidine 10^{-5} M (2) quinidine 5×10^{-5} M (3) Horizontal lines denote tetanic tension

in normal Ringer's were observed also in urea Ringer's but they were often more pronounced in the urea medium (Fig 4)

Drug effects on twitch and tetanus Chlorpromazine imipramine and quinidine all potentiated the twitch and prolonged its duration (Fig 5) The increase in twitch amplitude was dependent on the concentration of the drugs added and also on the initial twitch tetanus ratio of the individual fibre (Table II) In 26 fibres the mean value of this ratio was 47 per cent in normal Ringer's (range 30—67 per cent) The twitch potentiation induced by a drug in a given concentration was more pronounced at a low initial twitch tetanus ratio than at a high

The lowest concentrations of chlorpromazine which gave a twitch potentiation were in the range 1—3×10^{-6} M In a few fibres it was possible to use a concentration of 3×10^{-5} M without immediate blocking of the electrical activity At this high concentration no fully reproducible twitches could be obtained but an initial twitch tension of more than 90 per cent of the tetanic tension was sometimes recorded With continued stimulation however the twitch tension was successively diminished When stimulated locally observation in microscope then revealed that parts of the

TABLE II Effects of chlorpromazine imipramine and quinidine on the twitch tetanus ratio of single muscle fibres

Number of expts	Solution	Twitch tetanus ratio in per cent mean and range
4	Ringer's	56 6 (43 8–66 7)
	Chlorpromazine 5×10^{-6} M	68 4 (55 1–75 8)
4	Ringer's	49 0 (42 5 57 0)
	Chlorpromazine 10^{-5} M	75 2 (56 7–83 1)
6	Ringer's	39 1 (30 0–50 0)
	Imipramine 10^{-5} M	50 4 (45 0–59 6)
4	Ringer's	42 3 (30 0—50 0)
	Imipramine 5×10^{-5} M	72 8 (61 6–85 6)
5	Ringer's	42 4 (35 0–61 3)
	Quinidine 10^{-5} M	53 4 (45 3–64 5)
9	Ringer's	47 1 (31 0–61 3)
	Quinidine 5×10^{-5} M	70 1 ([illegible])

present results suggest that it is not necessary to disrupt the T system to diminish or eliminate the negative afterpotential. A loss of this potential could be demonstrated in urea Ringer's but it returned after changing to normal Ringer's at which time the contractility of the fibre was also normal.

Although urea in some fibres eliminated the negative afterpotential this action did not prevent the prolongation of the repolarization phase induced by chlorpromazine, imipramine and quinidine. Whether this drug effect is mainly exerted on the T system which is responsible for 2/3 of the potassium permeability of the fibre (Eisenberg and Gage 1969), the surface membrane or both cannot be concluded from the present experiments but requires further investigations on fibres with disrupted T tubules.

Drug effects on tension development. As is obvious from the present results chlorpromazine, imipramine and quinidine have similar effects on the electrical and mechanical activity of single muscle fibres. Kopera and Armitage (1954) found that chlorpromazine, after a transient potentiation, reduced the contraction response to both direct and indirect stimulation in the gastrocnemius muscle of the cat, and the same actions on frog sartorius (Isaacson and Sandow 1967) and on crab muscle (Huddart 1971) have been reported for quinine. Balzer and Hellenbrecht (1969) working with the sartorius and rectus abdominis muscles of the frog also found that chlorpromazine and quinidine caused a diminished tension output. The present results, however, clearly demonstrate that chlorpromazine $\leqslant 10^{-5}$ M, imipramine and quinidine $\leqslant 5 \times 10^{-5}$ M, concentrations that do not block the electrical activity, can potentiate the twitch response to direct stimulation of single muscle fibres. It is probable that some of these differences in results are due to the use of different preparations. However, in single muscle fibres it could be shown that 3×10^{-5} M chlorpromazine and concentrations of imipramine and quinidine exceeding 5×10^{-5} M caused a marked increase in the electrical threshold and greatly decreased the rate of rise and overshoot of the action potential. In a whole muscle these effects may lead to an incomplete activation of some of the fibres and in this way cause a diminution of both twitch and tetanus tensions. Supporting this explanation is the fact that the reduction of twitch amplitude reported by Balzer and Hellenbrecht (1969) could to some extent be counteracted by raising the strength of the stimulus.

At the high concentrations indicated above, the drugs used in this study sometimes caused an initial increase in the twitch tetanus ratio up to more than 90 per cent. During the following impulses, however, a decline in twitch tension was seen. The findings of (a) an initial increase in the peak twitch tension at high drug concentrations, (b) the persistence of a potassium contracture of unchanged amplitude at drug levels that blocked the electrical activity, and (c) the stable twitch potentiation seen at lower drug levels do not support the view of Balzer and Hellenbrecht (1969) that the amount of calcium released from the sarcoplasmic reticulum during activation is diminished, nor does the lowering of the mechanical threshold produced by these drugs (Andersson 1972 a) favour this suggestion. In a single fibre the reduc

tion of twitch response can be best explained by the demonstrated changes in excitability and the shape of the action potential which favour the formation of local conduction blocks This will lead to incomplete activation of the fibres and to reduced tension Etzensperger (1971 b) evaluating the mechanical response of individual fibres within small bundles of frog sartorius found that the presence of local anesthetics (cocaine and procaine) in the extracellular medium initially increased the twitch but subsequently decreased the response The decrease of tension began before any sign of disturbed activation could be seen in the microscope therefore it was suggested that this effect was due to a reduced effectiveness of a given depolarization to mobilize calcium Supporting this explanation was the finding that the local anesthetics used increased the mechanical threshold The same mechanism cannot explain the effects of chlorpromazine imipramine and quinidine Although these agents similar to conventional local anesthetics have membrane stabilizing effects (Shanes 1958 a b van Zwieten 1969 Langslet 1970 Langslet *et al* 1971) they lower the mechanical threshold and hence ought to increase the effectiveness of a given depolarization to mobilize calcium as suggested by Isaacson and Sandow (1967)

The present study demonstrated that the twitch potentiation induced by chlorpromazine imipramine and quinidine was accompanied by a prolongation of the active state duration This agrees with previous investigations on the effects of quinidine on whole muscle (Lammers and Ritchie 1955) Taylor Preiser and Sandow (1969) pointed out that as potentiators such as caffeine and nitrate enhance the rate of tension development very early in the twitch it is possible that these drugs accelerate the development of the active state No effect on the rising phase of the active state could be demonstrated in the present study but this may not be significant as Edman (1970) showed that the active state in a muscle fibre (at 1—2° C) required only 3—4 ms to rise from 25 to 65 per cent of its peak value With this short time interval it would be impossible with the techniques used in the present study to resolve such small changes in the build up of the active state that could be expected from the study of Taylor Preiser and Sandow (1969)

A prolongation of the active state duration with concomitant twitch potentiation can be produced in different ways (for reviews see Sandow 1965 Bianchi 1968)

1 by lowering the mechanical threshold i e the membrane potential level at which the mechanical activity is initiated
2 by increasing the mechanically effective period i e the time during which calcium is released from the sarcoplasmic reticulum
3 by inhibiting the uptake of calcium by the sarcoplasmic reticulum

There are reasons to believe that chlorpromazine imipramine and quinidine potentiate the twitch by all the mechanisms mentioned above Thus it has been demonstrated that these substances lower the mechanical threshold (Andersson 1972 a) inhibit the calcium uptake in the sarcoplasmic reticulum (Balzer Makinose and Hasselbach 1968 Worsfold and Peter 1970) and prolong the duration of the

action potential (Fig 1 and 2) Which of these effects is of greatest relevance for the twitch potentiation cannot be assessed at the present time

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Acta physiol scand 1973 88 342—345

From the Departments of Clinical Chemistry Lund and Vaxjo and the Department of Medicine (B) University Hospital Lund Sweden

On the Elimination of Transfused Heparin Induced Diamine Oxidase (DAO) Activity in Rabbits

By

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Abstract

Hansson R and H Thysell *On the elimination of transfused heparin induced diamine oxidase (DAO) activity in rabbits* Acta physiol scand 1973 *88* 342—345

Lymph or blood plasma rich in diamine oxidase (DAO EC 1 4 3 6) activity was obtained from donor rabbits after i v injection of heparin The lymph or after heparin neutralization the blood serum was injected into recipient rabbits and the elimination of the transfused DAO activity was studied Analogously to earlier findings in man the elimination rate of transfused DAO activity was several times more rapid than is indicated by the apparent rate of return to normal levels in rabbits after heparin induced increase in blood plasma DAO activity Furthermore our findings indicate the possibility of 2 different components of the elimination of transfused postheparin rabbit DAO activity somewhat contrasting to the findings in man

In all vertebrates examined i v injection of heparin is followed by an increase in the blood plasma diamine oxidase (DAO) activity EC 1 4 3 6 which describes one or more maximal value(s) before the final elimination slope (Hansson and Thysell 1968) It has been demonstrated that in man (Dahlbäck *et al* 1968) rat and rabbit there is a protracted infusion of lymph DAO the activity of which is 10—100 times higher than in the blood plasma more than 1 h after the heparin injection (Hansson and Thysell 1971) The possibility has been advanced that a delayed contribution from organs which are more remote targets to the heparin may lead to the second and major hump of the DAO (histaminase) curve in rabbits (Glanzmann and Glanzmann Zapel 1971) Whatever the reason may be there is a delayed contribution of DAO into the blood stream leading to an underestimation of the clearance of postheparin DAO from blood plasma In man the apparent elimination of DAO after heparin injection occurs with a half life of about 1 h for the liberated DAO (Hansson 1970) Transfused heparin induced DAO is however eliminated approximately 20 times faster (Hansson 1970) Similar data from other species seem to be lacking in the literature The rabbit exhibits the two humped type of postheparin DAO in

crease in plasma and furthermore an apparent eliminatory phase with a half time of about the same duration as that of man (Hansson 1970 Glanzmann and Glanzmann Zapel 1971) and was consequently chosen for further studies These include injection of DAO rich postheparin blood serum into healthy and liver in jured rabbits

Materials and methods

11 rabbits weighing 1 7 to 2 7 kg were used One rabbit was used as chylous lymph donor after heparin (for experimental details including DAO determination see Hansson and Thysell 1971) as a pilot test to assess the elimination of the DAO in lymph to the recipient rabbit (Table I no 1) Four rabbits were sacrified as donors of DAO rich plasma, by bleeding 75 min after the i v injection of 1 6 IU heparin (Vitrum Sweden) 6 further rabbits were used as recipient animals (no 2—7) two of which (5 and 6) had been injected with CCl_4 (0 1 g per kg b wt.) in soybean oil emulsion (Intralipid® Vitrum) 24 h prior to the DAO plasma transfusion The half life of injected sulfobromphtalein 5 mg per kg b wt increased from 7 25 and 7 75 min respectively before to 27 and 18 min after the CCl_4 injection at autopsy the livers showed a distinct nutmeg pattern To the rabbits with liver injury two injections of DAO rich blood serum with 20 min interval were given in order to assess the reproducibility of the procedure Finally one rabbit was given an infusion of DAO rich blood serum at the rates of (6 ml) 15 and (10 4 ml) 26 4 mU per minute respectively When steady state was obtained for the plasma concentration (at 0 175 and 0 300 U DAO/l recipient plasma) the fractional clearance could be estimated (Posen 1970) The declining phase after the infusion was also followed according to the rules for the single injections and the DAO values noted (Rabbit 7) All rabbits were provided with one cannula in one of the carotid arteries and one in the contra lateral jugular vein for sampling and injection respectively Local anesthesia was given prior to the cannulation (2—3 ml of 1 % Xylocain® solution Astra Sweden)

Results

Results are given in Table I The log DAO values (ordinate) were plotted versus time (abscissa) and the graphical peeling technique was used to determine the half life of the rapid first phase the basal level or slope was extrapolated towards zero time and subtracted from the total DAO values leading to a difference with approximately linear course

Comments

The results indicate that the half life of transfused postheparin DAO in the rabbit with the applied techniques varies around 0 7 min (Range 1 1—0 3 min) In this pilot study no hints were found that the addition of protamine or damage to the parenchymal liver cells influenced the disappearance rate of transfused DAO from plasma more than individual variation seems to do

In comparison to the findings in man (Hansson 1970) the disappearance of DAO from rabbit plasma seems to consist of at least one more component the peeling technique giving a seemingly rectilinear course of the log DAO This finding may be consistent with the possibility of more than one form of DAO (*op cit*) There is however one difference between the experimental conditions in the 2 species under comparison In the human experiments the injected volume did not exceed 8

TABLE I Clearance of transfused diamine oxidase (DAO) to rabbits Time zero indicates the mid infusion time in rabbits 1—6 but end of infusion in rabbit 7

Recipient rabbit no		1	2	3
Weight (kg)		2 7	1 7	1 7
Pretreatment		None	None	None
DAO vehicle/vol (ml)		Lymph/18	Serum/20	Serum/20
DAO content (mU)		125	37	61
Protamine addition U/ml		None	12	12
Value before injection		016	011	016
after	25 min		0 378	590
	50		674	473
	75		642	380
	1 0	223	[illegible]	286
	1 25		491	257
	1 50		516	243
	1 75		429	243
	2 00	339	419	222
	2 25		413	223
	2 5		347	219
	2 75		374	246
	3 0		386	193
	3 25		264	243
	3 5		293	215
	3 75		311	211
	4 0		280	226
	4 5		286	181
	5	102	250	200
	6		208	175
	7		—	[illegible]
	8		187	154
	10	032	172	132
	15		140	096
Calculated DAO half life min	Rapid phase	0 9	1 1	0 4
	Slow phase	4	7	9

cent of the plasma volume of the recipient whereas the rabbits received transfusion volumes approximately (Thysell 1972) amounting to 15 per cent of the plasma (Rabbit 1 with rather horizonal postelimination level) and about 25 per cent in the others (with their sloping tendency after the steep first part of the curve) In spite of rather constant DAO activity levels during the infusion (Rabbit 7) or injection the plasma volume may have increased and the process of elimination of DAO may have coincided with a redistribution of the exogenous protein rich fluid (Table I) Another approach to the problems of elimination of transfused postheparin DAO activity from the plasma thus seems to demand less crude preparations of the enzyme However our experiments indicate that the size of order of the fractional rate of total plasma clearance may be similar to that of man and tenfolds higher than indicated by the eliminatory phase of the DAO curve in the heparin stimulated animal

4 1 7 None	5 a 2 0 CCl	5 b 2 0 CCl	6 a 1 7 CCl	6 b 1 7 CCl	7 1 4 None
Serum/20 50 16	Serum/20 52 16	Serum/20 52 16	Serum/20 57 16	Serum/20 55 16	Serum/20 Σ = 140
009	006	031	026	013	—
558	450	503	526	520	300
348	465	541	373	467	—
231	415	383	326	331	177
201	339	289	275	248	—
155	297	251	206	196	099
101	296	283	188	210	—
081	235	203	163	155	066
063	247	215	141	153	—
051	—	235	—	—	046
050	192	186	086	131	—
038	—	197	—	—	038
042	143	150	095	132	—
029	—	—	—	—	032
036	—	135	—	—	—
032	—	—	—	—	040
029	119	143	079	—	—
028	—	—	—	098	035
026	072	137	064	112	028
020	—	—	—	—	027
019	079	094	054	082	022
021	—	—	—	—	028
021	049	073	038	059	—
020	—	054	—	—	—
0 4 31	0 9 9	0 8 10	0 3 10	0 3 12	5 >25

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Acta physiol scand 1973 88 346—349

From the Departments of Clinical Chemistry Lund and Vaxjo and the Department of Medicine (B) University of Lund Sweden

Effect of Organ Removal or Damage on the Heparin-Induced Diamine Oxidase (DAO) Response in Rat and Rabbit

By

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Received 6 October 1972

Abstract

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The postheparin DAO increase in blood plasma has been shown to depend on intact intestines in rat and rabbit Our findings indicate however that sources of mobilizable DAO may be found also outside the small intestine in these species In the rat after partial portal stricture for a week hepatectomy was followed by an intense and rather sustained post heparin DAO increase as compared to normal or shamoperated rats

The report on release after heparin injection of diamine oxidase (DAO) EC 1 4 3 6 from the liver in the guinea pig (Bernauer *et al* 1964) has been followed by communications concerning rat and rabbits In the rat removal of the intestine from the cardiac sphincter and to the coecum drastically reduced the rise in plasma diamine oxidase produced by heparin suggesting that the enzyme in the intestine is the main contributor to the raised plasma levels (Kobayashi 1969) In the rabbit total obstruction of the coeliac and both the mesenteric arteries caused a total extinction of the post heparin increase of this histaminase (DAO) activity 15 and 60 min after the injection (Glanzmann and Glanzmann Zapel 1971) However, damage to the transport routes of heparin to the target organs and DAO from them may influence the plasma DAO response to injected heparin

The intention of the present investigation is by more frequent sampling and more selective technique for the exstirpation (or damage) to study the remaining response of the heparin induced DAO increase in rat and rabbit plasma after organ exstirpation

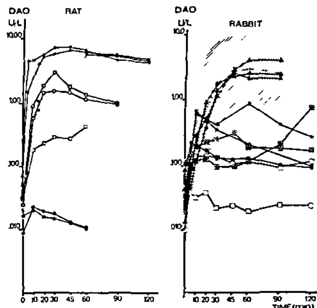


Fig 1 Effect of organ exstirpation or damage on the plasma DAO increase after heparin injection (at time zero) in the rat and the rabbit Symbols Shaded area for normal range Filled triangles nephrectomy filled stars CCl_4 damage (rabbits) or hepatectomy (rats) open stars in squares colectomy (rabbits) squares exstirpation of the small intestine filled circles total enterectomy Heparin dose at time zero 1 6 U/g b wt

Material and methods

The experimental routine with respect to drugs animals cannulation injections sampling and determination of diamine oxidase activty was the same as previously described (Hansson and Thysell 1968 1969 1971)

In addition the following operative p ocedures were used

Rat Both sexes weight 160—500 g 1 Portal vein stricture and hepatectomy Access to the portal vein was achieved by a median abdominal incision The vessel was dissected free and partially obstructed by l gature The abdomen was closed by suture of the peritoneal sac the fascia and the skin After a week the hepatic vein and artery were identified and simultaneously clamped and l gated The liver was excised carefully so that the mesentery its root and lymphatics were not damaged The abdomen was then closed by sutures or forceps ? Enterectomy Access to the abdomen was similar to 1) Pylorus was dissected free With ligatu es placed as closely to the vi cus as possible (so as not to interfere with the larger lacteals in the mesenteric root) the whole intestine was excised from the pylorus and to the ampulla r ct The abdomen was then closed

Rabbit Both sexes weight 1 7—3 0 kg 1) After 7 days of partial portal stricture hepatectomy was tried but always failed due to profuse local bleedings Instead of hepatectomy therefore *hepatic damage* was p ovoked by injection into an ear vein of carbon tetrachloride 0 1 ml per kg b wt distributed n about 2 ml of 10 % (w/v) soybean oil emulsion (Intralipid® Vitrum Sweden) 2) Ent rectomy Similar techniques were used as for the rat When the caecum plus colon or the small intestine was x t rpated sepa ately due cons de ation was given to the circulation of the remaining intestinal segm nts because of the intimate conne ions of their vascular areas 3 Nephrectomy (see Thysell 1972) Retroperitoneal app oach under local anesthesia (< 2 ml Xylocain® 1 Astra) ty ng of the renal stalk and removal of the kidney bilaterally in one session

Only animals in good condit on du ng a po top rative period of mo e than one hour were used fo further expe i nents

Results and comments

The results are given in Fig 1

In rats an abdominal sham operation does not seem to diminish the plasm response to heparin (Hansson and Thysell 1971)

Acta physiol scand 1973 88 346—349

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The intention of the present investigation is by more frequent sampling and more selective technique for the extirpation (or damage) to study the remaining response of the heparin induced DAO increase in rat and rabbit plasma after organ extirpation

reactive pattern after selective evisceration indicating the intestines as the main source of the liberated enzyme Intact rats and rabbits typically exhibit a similar blood plasma DAO increase consisting of two ascending phases (Hanson and Thysell 1968 and 1970) after heparin injection in contrast to the guinea pig with its early maximum and only one obvious ascent They also differ concerning their mighty lymph plasma gradient of DAO from the guinea pig with its lower simultaneous DAO values in lymph than in plasma In all these species however the mobilizable DAO (histaminase) activity is available for release into the blood compartment as described in anaphylactic shock by Rose and Leger by Code and by Logan (for ref see Hansson and Thysell 1968) or after heparin administration (Bernauer 1964 Hansson and Thysell 1968) indicating a DAO reactive pattern with a possibly ameliorating effect on the consequences of the shock

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Protein Concentration of Interstitial Fluid Collected from Rat Skin by a Wick Method

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Abstract

AUKLAND K and H O FADNES *Protein concentration of interstitial fluid collected from rat skin by a wick method* Acta physiol scand 1973 88 350—358

A new method has been developed for studying interstitial fluid protein concentration A 3 cm long and 0 5 mm thick nylon thread was sewn into subcutaneous tissue of ether anesthetized rats and left for equilibration with interstitial fluid for 35 to 240 min The wick was then pulled out both ends cut off and the middle part quickly transferred to a tared vial containing 2 ml saline and weighed Blood stained wicks about 1 out of [illegible] were discarded After 24 h elution the wick was removed dried and weighed allowing calculation of wick fluid volume Total protein albumin and hemoglobin concentrations were measured in the eluate Hemoglobin concentration was less than 0 2 g/100 ml in all but one wick Implantation of 73 wicks in 24 rats for 35 to 1[illegible]0 min gave an average albumin concentration of 2 10 (S D 0 [illegible]4) g/100 ml or 63 % of plasma albumin concentration Total protein concentration in 80 wicks averaged 3 44 (S D 0 30) g/100 ml 56 % of plasma The concentrations did not change during this time interval and were reduced by less than 10 % by antihistamine/antiserotonin treatment An irregular rise in concentrations after more than 2 h implantation was delayed by antihistamine/antiserotonin

The concentration and composition of proteins in plasma and interstitial fluid determine the net oncotic pressure across the capillary walls and are thereby of great importance for transcapillary fluid balance Estimates of interstitial fluid protein concentration have been obtained by measuring 1) whole body serum protein content plasma volume and extracellular volume 2) protein concentration of lymph 3) plasma protein content plasma volume and extracellular space of excised tissues 4) protein concentration of fluid collected directly from the tissue

While the first approach gives an average for interstitial fluid of all organs and tissues lymph samples may be obtained from limited regions but will often represent a mixture of lymph from different tissues for instance skin and muscle and do not necessarily represent average interstitial fluid protein concentration Furthermore to obtain sufficient samples it is often necessary to stimulate lymph flow by heating passive movements or other procedures which might well influence the protein concentration Measurements on excised tissues possibly represent the best available

data on local interstitial fluid protein concentrations so far but involve considerable methodological problems especially the determination of extravascular protein space (Katz *et al* 1970) Edema fluid is easily available for local sampling whereas only few measurements have been made on normal interstitial fluid

Inspired by Scholander's wick method for measuring interstitial fluid pressure (Scholander, Hargens and Miller 1968) we have attempted to estimate interstitial fluid protein concentration by measuring the concentration of the fluid contained in a wick after implantation The method is direct and simple and would seem applicable to many problems concerning capillary permeability and effective capillary oncotic pressure provided it can be shown that the wick fluid represents normal interstitial fluid with respect of protein content Indirect evidence for this assumption will be presented in the following report on measurements in subcutaneous tissue of rats Preliminary experience with the method has been presented previously (Aukland and Fadnes 1972)

Methods

Wick technique Wicks were made from about 0 1 mm thick nylon thread containing 75—100 fibers (Pearsalls No 3 Twine Bonded James Pearson & Co Taunton Sommerset, England) Six such threads were twisted together and manipulated such as to loosen the fairly firm texture of the thread and divided in 8 to 10 cm long pieces A knot was tied on one end and the other end was thread onto a 4 cm long thin, round and slightly blunted surgical needle Before introduction the wick was soaked in 0 9 % saline Wistar rats of either sex weighing about 250 g were used for all experiments They were anesthetized with ether and the skin over the lower back was shaved 3—6 wicks were inserted in each animal by stitching the needle under the skin for about 3 cm across the back of the animal and pulling the wick gently through until arrested by the knot The needle was then cut off and the rat returned to its cage where it recovered from anesthesia in the course of 15 to 30 min One half to four hours after insertion the wicks were pulled out. Both ends were cut off and the middle section about 2 cm long was quickly transferred to a test tube containing 2 ml 0 9 % NaCl The tube was weighed to the nearest 0 1 μg immediately before and after addition of the wick permitting calculation of the weight of the fluid filled wick The whole procedure exposed the wick and eluating fluid to open air for only about 5 s The tube was shaken vigorously and left at room temperature for 24 h After repeated shaking the wick was removed dried for at least 4 h and weighed allowing calculation of the weight of the wick fluid Assuming a specific weight of 1 00 the fluid volume usually ranged from 3—5 μl Protein concentrations were thereafter determined in the 2 ml eluate Wicks with obvious blood staining were discarded

Protein determinations Total protein was estimated by the Amidoschwartz method of Munk Plum *et al* (1955) as modified for small protein quantities by Aukland (1960 To 0 50 ml of sample was added 0 2 ml of precipitation reagent (12 5 mg Amidoschwartz 10 B in 100 ml citric acidphosphate buffer) After thorough mixing the tube was left for 15 min centrifuged for 15 min at room temperature at 3000 r p m and 0 25 ml of the supernatant was pipetted off and diluted with 0 50 ml distilled water Optical density was then read against distilled water in an Unicam SP 800 Ultraviolet Spectrophotometer at 600 mμ using 1 cm microcuvettes Standard dilutions of rat serum containing 50 and 100 μg protein per ml were included in each run The method was calibrated on rat serum and a standardized human serum (Seronorm Nyegaard & Co AS Oslo) against the biuret method of Klungsøyr (1969) Since more Amidoschwartz is bound to albumin than to globulins calibration against Klungsøyr's method was also performed on concentrated wick eluate obtained by eluating 5—6 wicks in 2 ml saline No significant difference from calibration on serum was observed

Albumin was determined spectro-fluorimetrically by ANS (1 Anilino naphthalene 8 sulphonic acid) principally as described by Rees Fildes and Laurence (1954) 0 1 ml wick eluate was mixed with 2 ml 25 μM ANS in 0 1 M phosphate buffer pH 7 5 The fluorescence intensity was measured in quartz cuvettes at 490 mμ with a Farrand Mark I spectrofluorometer equipped with an arc stabilizing device using an exciting beam of 390 mμ Standard solutions prepared from rat albumin (Fraction V Powder Sigma Chemical Comp St. Louis Mo USA)

TABLE I. Protein recovery from wick

	Total protein mg/100 ml	%	Albumin mg/100 ml	%
Diluted serum*	3.00	100	1.70	100
Wick no. 1	3.02	101	1.73	101
2	2.78	93	1.77	104
3	3.00	100	1.71	100
4	3.11	104	1.8?	107
Mean of wicks	2.98	99	1.75	103

* Rat serum diluted with equal volume of saline.
Incubation time 40 min.

run repeatedly in each series. The contribution to fluorescence in serum samples by non-albumin proteins is negligible compared to that of albumin (Rees et al. 1954, Dillard et al. 1971). Washed and hemolysed red cells were found to bind ANS in amounts corresponding to an albumin concentration of less than 5% of the hemoglobin concentration.

Total protein and albumin determinations were run in duplicate on all wick eluates. Measurements were also made on serum from 10 rats obtained by cutting the femoral artery.

Hemoglobin concentration was determined in 56 wick eluates by a Benzidine method (Crosby and Furth 1956) as modified for low concentrations. To tubes containing 1 ml Benzidine solution (4 g/100 ml in 90% acetic acid) was added 0.4 ml sample and 1 ml 1% H_2O_2. After 20 min at room temperature, 4 ml 10% acetic acid was added, and exactly 5 min thereafter optical density was read at 510 mμ against reagent blank. Standards containing 0.1—1 mg/100 ml were made from whole blood of known hemoglobin concentration (determined as cyanmethemoglobin) by washing and subsequent freezelysis of the erythrocytes. Ghosts were not removed.

Experimental design. In vitro recovery of total protein and albumin was tested by soaking wicks in rat serum diluted with equal volume of saline. After equilibration for 15—60 min the wicks were pulled out. Excess fluid was wiped off and the wicks transferred to tared vials as described for implanted wicks.

Control group. A total of about 150 wicks were sewn into subcutaneous tissue in 24 rats and removed after 1/2 to 4 h. About 1/5 of all wicks were blood stained and were omitted from further analysis.

Antiserotonin/antihistamine group. 3 rats were given 100 μg cyproheptadine chloride in 0.1 ml water intraperitoneally, and the wicks were soaked in the same solution before implantation. Similarly, 11 rats received 25 μg methysergide in 0.5 ml i.p. and wicks soaked in methysergide solution 50 μg/ml. The i.p. injections were given 30 min before wick implantation.

Histamine group. In 3 rats 9 wicks soaked in 1% histamine chloride solution were implanted for 1 h. The initial histamine content of each wick can be estimated to 30—50 μg.

Results

In vitro test. Incubation of wicks in rat serum diluted with saline 1:1 for at least 30 min and subsequent analysis showed good agreement with protein determination directly on serum. Wick fluid volume was comparable to that obtained after s.c. implantation. Table I, showing the results of one such experiment, also demonstrates the fairly good reproducibility of the whole procedure. Incubation of wicks for 15 min showed considerably lower concentration in wick fluid, indicating incomplete equilibration.

Serum protein concentrations. Total protein concentration determined in 14 rats averaged 6.12 g/100 ml (S.E. 0.19) with a range of 5.4 to 7.4 g/100 ml. Albumin

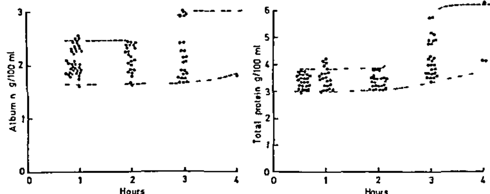


Fig 1 Protein concentration in wick fluid after subcutaneous implantation for 35 to 240 min Left Albumin concentration Right Total protein concentration More than 90 % of the values are confined within the broken lines drawn to best visual fit

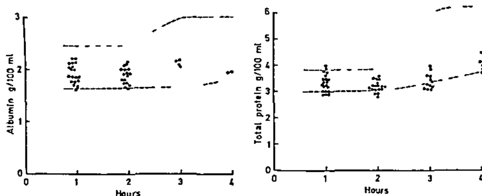


Fig 2 Protein concentration in wick fluid after subcutaenous implantation in rats pretreated with cyproheptadine or methysergide Left Albumin Right Total protein Broken lines as in Fig 1 including more than 90 % of control values

concentration averaged 3 34 g/100 ml (S E 0 08) with a range of 2 9 to 4 0 g/100 ml

Blood contamination About 1 of 5 wicks was obviously blood stained and was discarded from further analysis Hemoglobin determination on 56 accepted wicks showed wick fluid concentrations of less than 0 1 g/100 ml in 43 wicks 0 11 to 0 20 in 12 and in one wick 0 4 g/100 ml

Wick fluid protein concentrations

Control group Total protein concentration was measured in 121 wicks implanted for 35—240 min As evident from Fig 1 the concentration showed no obvious chan e

TABLE II Serum and wick fluid protein concentrations after 35–120 min implantation

	Number of rats	Total protein g/100 ml				Albumin g/100 ml			
		n	Mean	S E	Range	n	Mean	S E	Range
Wick fluid									
Control	24	80	3 44	0 03	3 00–4 17	73	2 10	0 03	1 62–2 51
Methysergide cyproheptadine	15	42	3 29*	0 05	2 77–4 00	44	1 92	0 06	1 50–2 23
Histamine	3	9	5 34*		3 80–5 89	10	2 74*		2 35–3 57
Serum	14		6 12	0 19	5 40–7 40		3 34	0 08	2 90–4 00

S E = Standard error of the mean
n = Number of wicks
* Significantly different from control $p < 0.02$

during the first 2 h of implantation averaging 3 44 g/100 ml (S D 0 30) or about 56 % of total plasma protein concentration More than 90 % of the values were within the limits 3 0 and 3 8 g/100 ml After 3 and 4 hours implantation average protein concentration rose significantly and the scatter increased considerably Fig 1 shows a similar pattern for albumin concentration During the first 2 h the average albumin concentration was 2 10 g/100 ml (S D 0 24) or 63 % of plasma albumin concentration with more than 90 % of the values within the range of 1 7 and 2 4 g/100 ml Also albumin concentration rose slightly after 3 h implantation (2 24 g/100 ml $p < 0.05$)

Antiserotonin/antihistamine group No difference was discernible between animals treated with methysergide or cyproheptadine and they were therefore treated together As evident from Fig 2 both total protein and albumin concentration showed great overlapping with control observations However in the period of 30–120 min after implantation average total protein concentration was reduced to 3 29 g/100 ml (95 % of control) and albumin concentration to 1 92 g/100 ml (91 % of control) both differences being statistically significant at the 2 % level (Student's t test) Furthermore the total protein concentration after 3 h implantation (3 49 g/100 ml S D 0 31) was considerably less than in control animals ($p < 0.01$) whereas albumin concentration (2 07 g/100 ml S D 0 24) was only slightly lower than control $0.1 < p < 0.2$) After 4 h implantation the antihistamine/antiserotonin treatment seemed to make little difference

Histamine group All wicks soaked in histamine solution before implantation showed much higher albumin and total protein concentration than obtained in control animals (Table II) reaching an average of about 85 % of plasma concentrations

All data obtained with 35–120 min implantation in the different groups are summarized in Table II including mean and standard error of the mean

Discussion

Recovery tests *in vitro* showed that representative samples of fluid can be collected and analysed by the wick method described above (Table I) The crucial question is therefore whether the fluid contained in wicks after subcutaneous implantation is representative for normal interstitial fluid with respect to protein concentration Too high values might result if insertion of the wick produced bleeding or local inflammation with increased capillary permeability to proteins To the contrary hyperemia with increased capillary pressure without change of capillary permeability might produce increased filtrate with protein concentrations lower than normal

The influence of blood contamination seems most easily excluded in more than 3/4 of all wicks without obvious blood contamination the hemoglobin concentration was less than 0 1 g/100 ml corresponding to a dilution of whole blood of the order of 1 100 With a total blood protein concentration of 20 g/100 ml this could increase total protein content in the wick fluid by only 5 % The influence on measured albumin concentration should be even less $< 1\%$ (see Methods) Because of the small influence of blood contamination estimated from these data no correction has been made in the presented figures However such estimates might be too low since the erythrocytes of extravasated blood might not enter the wick to the same extent as plasma proteins This possibility can neither be excluded nor confirmed by the present experiments but the rather small variation between different wicks strongly suggests that bleeding cannot be a major disturbing factor

The possibility of changes in interstitial fluid protein concentration due to hyperemia or increased capillary permeability is more difficult to rule out but the results presented above give some indirect evidence

1) The modest scatter amongst wicks implanted for 30—120 min and thet constancy of average protein concentration are difficult to reconcile with appreciable changes taking place in this period A possible change in interstitial fluid protein concentration would therefore have to be much the same in all wicks and would also have to take place during the first half hour after implantation This possibility cannot be tested directly because a minimum of half an hour is needed for complete equilibration with surrounding fluid and we therefore attempted to block or minimize possible inflammatory reactions

2) Local and general treatment with methysergide or cyproheptadine reduced average concentration of albumin and total protein by less than 10 % Though statistically significant this effect does not suggest any major changes taking place due to histamine or serotonin release during the first 1/2 h after implantation This finding may well be due to a lucky choice of experimental animal since the initial inflammatory increase in vascular permeability to thermal injury is much smaller in rats than in guinea pigs and rabbits (Wilhelm and Mason 1960) However an early inflammatory reaction may be induced in rats by other stimuli and both histamine and serotonin have been advanced as mediators (for references see Spector and Willoughby 1963 Movat 1971) This was the basis for selecting cyproheptadine with combined antihistamine/antiserotonin effect and methysergide which is a strong serotonin antagonist

Although the modest effect of methysergide and cyproheptadine suggests a sup pression of an inflammatory increase of capillary permeability to proteins caused by wick insertion a direct effect of these agents on capillary permeability or capillary pressure cannot be excluded Furthermore it is obvious that a significant dif ference of the observed magnitude (< 10 %) might well result from uncontrolled factors such as seasonal variations differences between groups of animals or un noticed changes in techniques

It seems more safe to assume that the marked rise in total protein concentration after 3 h implantation (Fig 1) is caused by an inflammatory reaction which can be delayed but not prevented by antihistamine/antiserotonin treatment (Fig 2) It was unexpected however to find less increase in albumin than in total protein concen tration While the cause of this discrepancy remains obscure the slight rise in al bumin concentration in control animals may well explain the failure of demonstrating significant effect of antihistamine/antiserotonin on 3 h albumin concentration

An acute inflammatory reaction due to formation of vasoactive peptides has not been excluded by the present experiments but again any such effect would have to take place within the first 1/2 h after implantation and affect all wicks approxi mately to the same extent

The possibility that ether anesthesia during wick implantation may have sup pressed an acute inflammatory response (Miles and Miles 1952) seems unlikely because of its short duration Furthermore preliminary experiments with implanta tion in unanesthetized rats have shown an average total protein concentration of 62 % (7 wicks in 3 rats range 58—71 %) of serum concentration not significantly different from the present control group (Fadnes unpublished results)

3) The present results compare favourably with several previous estimates of interstitial fluid protein concentration Direct measurement of albumin content of human skin together with assumed interstitial fluid volume has shown interstitial concentrations of 45 % of plasma concentration (Rotschild *et al* 1956) More com plete analysis of rat skin by Katz *et al* (1970) including measurements of extracel lular space and correction for intravascular protein showed an average albumin concentration of 2 0 g/100 ml or 61 % of that in plasma in good agreement with the present results Practically the same albumin concentration (1 9 g/100 ml) was obtained in rat skeletal muscle whereas Creese D Silva and Shaw (1962) found somewhat lower albumin concentration (42 % of plasma) in fluid collected by micropuncture of guinea pig skeletal muscle

The considerably lower albumin and total protein concentrations in interstitial fluid usually inferred from protein concentration in extremity lymph may have several explanations In the past many authors assumed that lymph was drained from interstitial fluid concentrated by water reabsorption at the venous end of the capillaries According to this theory lymph protein concentration would be ex pected to exceed the average concentration of interstitial fluid However recent kinetic studies on paw lymph in dogs by Garlic and Renkin (1970) strongly suggest that there are no substancial gradients of concentration within the interstitial

space beyond the blood capillary walls Since the same investigators found no exchange of water or macromolecules across the walls of the lymph vessels it follows that lymph should equivalate average interstitial fluid as far as protein concentrations are concerned However this only means that lymph is representative for interstitial fluid under the circumstances of the experiment and it seems well established *that procedures used to increased lymph flow for easier collection reduce* protein concentration (Drinker and Field 1933 Renkin 1964) The importance of lymph flow rate is especially well illustrated in the experiments on dog paw by Garlic and Renkin (1970) where spontaneous variations in lymph flow (under passive movements of the paw) resulted in variations of lymph/plasma ratios for albumin from 0 1 to 0 7 The ratios obtained at low flows 0 5 to 0 7 agree well with the present average of 0 63

The protein concentrations of interstitial fluid observed in the present study should correspond to a colloid osmotic pressure of about 10 mm Hg (Landis and Pappenheimer 1963) This is considerably higher than the average value of about 4 mm Hg usually found in the free fluid of the tissue space as stated in a recent review by Guyton Granger and Taylor (1971) As outlined above this statement may well be due to a misinterpretation of lymph data and it is noteworthy that direct estimates on rabbit skin by Wiederhielm Stromberg and Lee (1970) showed colloid osmotic pressures of 8—15 mm Hg Since the protein concentration will fall with increased net capillary filtration and lymph flow it is obvious that an interstitial fluid colloid osmotic pressure of this magnitude will represent a powerful buffer against edema formation This mechanism has recently been emphasized by Wiederhielm (1968) who showed with an analogue computer program that an interstitial colloid osmotic pressure of 10 mm Hg is well compatible with available data on other factors governing transcapillary fluid balance

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Sequential Exocytosis of Storage Granules during Antigen Induced Histamine Release from Sensitized Rat Mast Cells in vitro
An electron microscopic study

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Abstract

ANDERSON P S A. SLORACH and B UVNÄS *Sequential exocytosis of storage granules during antigen induced histamine release from sensitized rat mast cells in vitro* Acta physiol scand 1973 *88* 359—372

The morphological changes appearing in sensitized rat mast cells incubated with antigen at 25 C for times ranging from 30 s to 10 min were studied using light and electron microscop Histamine release was assayed in parallel After a latent period of 30 s during which no histamine release or morphological changes occurred degranulation and histamine release commenced and increased progressively with time The first changes were seen in the most peripherally located granules and consisted of a fusion of the cell membrane with the perigranular membrane and a swelling and a reduction of the electron-density of the granules With increasing time these changes were seen to spread deeper into the cells resulting in the formation of labyrinthic cavities containing changed granules such granules were also seen outside the cells Using the extracellular tracer lanthanum these apparently intracellular cavities were demonstrated to be in unbroken communication with the extracellular milieu Thus when sensitized rat mast cells are incubated with antigen sequential exocytosis of histamine storing granules take place The results agree with the hypothesis that histamine release takes place by ion e change between histamine and extracellular cations both in granules expelled from the cells and in those retained in cavities open to the exterior

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Histamine is released when mixed peritoneal cell suspensions (Garcia Arocha 1961) or isolated peritoneal mast cells (Uvnas and Thon 1959 Perera and Mongar 1963 Johnson and Moran 1969) from sensitized rats are incubated with antigen Anderson and Uvnas (1971) found evidence for a sequential exocytosis of histamine release from mast cells incubated with another releaser compound 48/80 Rohlich Anderson and Uvnas (1971) found evidence for a sequential exocytosis of histamine storing granules These findings were in agreement with the hypothesis of Uvnas (Uvnas 1964 Uvnas and Thon 1966 Thon and Uvnas 1967) that during this exocytosis histamine is released from its binding sites in mast cell granules in exchange for cations in the extracellular medium Histamine will be released from both expelled granules and from granules retained in the extracytoplasmic cavities w

are in open connection with the extracellular space observed during the degranulation process (Rohlich *et al.* 1971).

The present study was undertaken to find out if a similar process of sequential exocytosis of histamine-containing granules occurs during histamine release in sensitized rat mast cells incubated with antigen. Since at 37° C histamine release in antigen challenged sensitized mast cells starts already after 10—15 s and is maximal after 30—60 s (Bloom and Chakravarty 1970) an incubation temperature of 25° C was used in order to retard the release and thereby facilitate the study of the initial ultrastructural changes occurring during histamine release. In order to minimize morphological changes during handling of the mast cells prior to incubation with antigen, mixed cell suspensions from peritoneum and pleura of sensitized rats were used.

Methods

Sensitization and preparation of cell suspension

Male Sprague Dawley rats (180 g) were sensitized by s.c. injection of 2 × 0.5 ml of egg albumin solution (100 mg/ml) and 2 × 0.5 ml of pertussis vaccine as adjuvant. 3—6 weeks later the rats were exsanguinated under light ether anesthesia by cutting the carotids and a small incision was made along the midline of the abdomen. 9 ml of a salt solution (NaCl 130 mM, $CaCl_2$ 2.0 mM) buffered with 10 % v/v Sorensen buffer (KH_2PO_4 + Na_2HPO_4 67 mM) pH 6.7 and containing 0.1 mg of human serum albumin per ml was pipetted into the peritoneal cavity. After massaging for 90 s the cell suspension was collected from the cavity. Cells were collected from the pleural cavity in an analogous fashion using 4.5 ml of the buffered salt solution. The cell suspensions were centrifuged (200 × *g*, 5 min, 2? C) and the resulting sediments resuspended in the above mentioned salt solution and mixed.

Incubation procedure

Samples for electron microscopy and for histamine assay were run in parallel. 0.9 ml aliquots of a cell suspension obtained from 12 rats (2—3 × 10 mast cells/ml) were preincubated at 25° C for 5 min before adding 5 g of egg albumin dissolved in 0.1 ml of buffered salt solution (see above); controls were incubated with 0.1 ml of the salt solution. Incubation was stopped after 30, 60, 90, 180 or 600 s by adding either 9 ml of cold (4° C) 2 % glutaraldehyde in Millonig phosphate buffer ($NaH_2PO_4 \cdot 2H_2O$ 134 mM, NaOH 107 mM) pH 7.3 (electron microscopy) or 9 ml of ice cold buffered salt solution (histamine assay).

Histamine assay

The samples were centrifuged (200 × *g*, 5 min at 4° C), the supernatants removed and each sediment then suspended in 10 ml of 0.1 N HCl to release the remaining histamine from the mast cells. Histamine was measured fluorimetrically according to the method of Shore, Burkhalter and Cohn (1959) as modified by Bergendorff and Uvnäs (1972). The histamine release was calculated as a percentage of the total histamine content of the cells.

Electron microscopy

Fixation was carried out for 5—10 min at 4° C and then continued for 2 h at 2? C. The cell suspensions were centrifuged (200 × *g*, 5 min, 2? C) and the sediments washed 3 times with Millonig phosphate buffer pH 7.3 containing 3 % sucrose. After postfixation in 1 % OsO_4 in Millonig phosphate buffer for 40 min at 4° C and centrifugation the sediments were washed with 0.1 M acetate buffer pH 5.0 for 10 min at room temperature. The cells were then contrast stained with 0.5 % uranyl acetate in the acetate buffer for 1 h at 2? C and again washed in the acetate buffer. After centrifugation each sediment was suspended in 5 drops of a warm freshly prepared 1? % gelatin solution and transferred to presiliconized polyallomer tubes and centrifuged at 1200 × *g* for 10 min at room temperature. The resulting pellets were taken out, sliced, dehydrated in an ethanol series, soaked in propylene oxide and embedded in Araldite.

The blocks were cut on a Reichert ultramicrotome Om U3 and the sections were stained with uranyl acetate and diluted lead citrate and observed in a Philips EM 300 electron microscope at 80 kV.

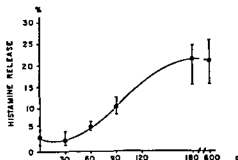


Fig 1 Time course of histamine release from sensitized rat mast cells incubated with antigen (5 μg egg albumin/ml) at 25 C Means of 4 expts Vertical bars indicate range

For light microscope observations semi thin sections were stained with a solution of toluidine blue and azure A containing 60 % sucrose

Tracing with lanthanum nitrate

0 9 ml aliquots of a mixed cell suspension obtained from 5 sensitized rats were incubated with 5 μg of egg albumin at 25 C as described earlier The release process was stopped after 30 60 120 or 180 s by diluting with cold (4 C) 2 glutaraldehyde in Millonig phosphate buffer pH 7 3 Controls without antigen were run in parallel After the initial fixation for 10 min at 4 C fixation was continued for 2 1/4 h at 22 C The cells were washed 3 times with Millonig phosphate buffer pH 7 3 and resuspended in 1 % lanthanum nitrate in 0 15 M cacodylate buffer pH 7 6 for 30 min at 22 C The cells were washed in 0 15 M cacodylate buffer pH 7 6 and resuspended in 5 drops of a warm freshly prepared 12 % gelatin solution The dehydration and embedding procedure was the same as described earlier

Thin sections cut on a Reichert ultramicrotome Om U3 were observed in a Philips EM 300 electron microscope at 80 kV without contrast staining

Materials

Egg albumin Difco Detroit Michigan USA Pertussis vaccine (10^9 bact /ml) SBL Stockholm Sweden Human serum albumin (free from preservatives) AB Kabi Stockholm Sweden Glutaraldehyde (25 % aqueous solution specially purified for use in electron microscopy) TAAB Laboratories Reading England

Results

Histamine release and light microscopy

The time course of histamine release from sensitized rat mast cells from peritoneum and pleura treated with antigen (5 μg egg albumin/ml) *in vitro* at 25° C is shown in Fig 1 The histamine release began after a latent period of 30 s and increased slowly to a mean maximal release of 21 % after 3 min The low concentration of antigen used (5 μg/ml) gave consistent results in the 4 expts performed (3 in parallel with electron microscopy)

During the first 30 s (Fig 2 a 2 b) no changes in the morphology of the mast cells could be detected in the light microscope the granules were stained dark blue with a toluidine blue azure A solution After incubation for 60 s some mast exhibited granules which were stained pink instead of dark blue with the

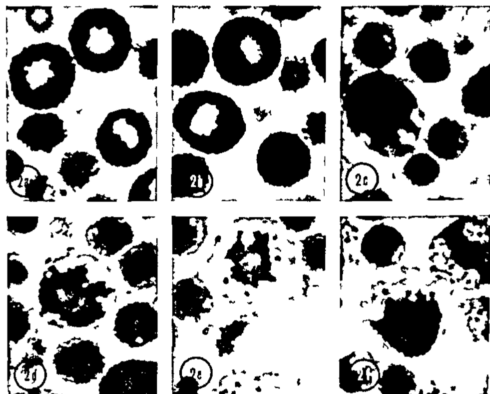


Fig 2 Light microscopy of (a) sensitized mast cells not treated with antigen (b) sensitized mast cells treated with antigen (5 μg egg albumin/ml) at 25 C for 30 s (c) for 60 s (d) for 90 s (e) for 180 s and (f) for 600 s Note the gradual degranulation starting from the periphery of the cells ×1700

tion similar granules could also be seen outside the cells (Fig 2 c—2 f) These changes increased with time and were correlated to the histamine release

Electron microscopy

Sensitized mast cells not treated with antigen

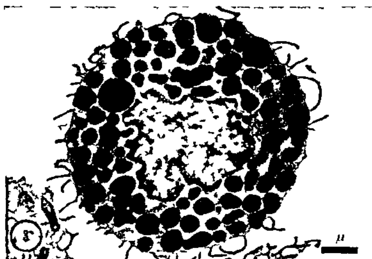
Sensitized mast cells not treated with antigen (egg albumin) showed the same morphology as normal mast cells which has been described earlier (Bloom and Haeger

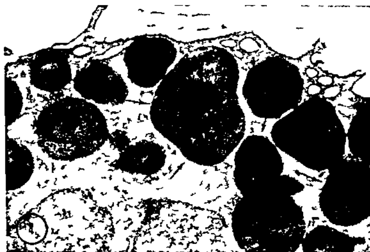
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Fig 3 Electron micrograph of a sensitized untreated mast cell Many homogeneous electron dense (osmiophilic) granules are seen in the cytoplasm ×9000

Fig 4 A higher magnification of a sensitized untreated mast cell showing homogeneous osmiophilic granules surrounded by perigranular membranes (arrows) In the cytoplasm rough and smooth endoplasmic reticulum and occasional mitochondria are also seen Part of the nuclear envelope surrounding the nucleus can be discerned ×26 000

Fig 5 Sensitized mast cell treated with antigen for 60 s The initial changes are observed in the most peripherally located granules and consist of granule swelling and loss of electron density Other granules inside the cell and other components of the cytoplasm are not affected Note the granule on its way out of the cell (arrow) and granules already released from mast cells ×9000







mark 1965 Horsfield 1965 Singleton and Clark 1965 Combs 1966 Rohlich *et al* 1971) All mature granules in the sensitized untreated mast cells are very homogeneous and electron dense (Fig 3) The aggregates of dense material in the granules of very young mast cells called progranules, are very easily distinguished from these mature granules The granules are surrounded by a perigranular membrane which can be discerned when sectioned transversly (Fig 4) No connections were seen between the perigranular membranes of neighbouring granules

Sensitized mast cells treated with antigen

Sensitized mast cells incubated with antigen (egg albumin) *in vitro* at 25° C did not show any evident morphological changes during the latent period before the histamine release

At 60 s when the histamine release had started very few mast cells showed ultrastructural changes The initial changes were almost always observed in the most peripherally located granules (Fig 2 c and 5) when looking at mast cells sectioned through their largest diameter The changes consisted of a swollen appearance and a reduction in electron density of the granules (altered granules according to Bloom and Haegermark 1965) when compared to normal granules At favourable planes of sectioning an apposition and a fusion of the cell membrane and the perigranular membrane could be observed (Fig 6) At such places the normal trilaminar structure of both membranes sometimes disappeared leaving behind a thin structureless electron-opaque diaphragm Such apposition and fusion of the cell membrane and the perigranular membrane could also be seen between the membrane of the cavities containing altered granules and the perigranular membrane of adjacent granules (Fig 7 At this stage it was also possible to observe granules on their way out of the cells (Fig 5) All other granules inside the mast cells seemed to be unchanged

At 90 s more granules showed the less electron dense swollen appearance of altered granules which are presumed to have released their histamine (Fig 8) Many of the changed granules were situated in membrane bounded cavities which at favourable planes of sectioning showed a communication to the extracellular milieu

At 3 min and 10 min the fusion process could be seen in more mast cells and to spread more deeply into individual mast cells and/or laterally so that granules lying in the vicinity of already changed granules were consecutively affected (Fig 9 10 and 11) In this way complicated labyrinthic cavities were formed containing many altered granules Some granules could be seen on their way out of the cells (Fig 9 double arrows) to the extracellular space where already released granules could be found

Granules with an electron density between that of normal granules and that of altered granules could sometimes be seen (Fig 10 marked X) These were always situated close to the cell periphery or close to cavities containing altered granules Such granules are assumed to represent granules in the process of swelling





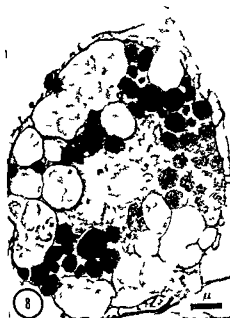


Fig 6 High power micrograph of an apposition and fusion of the perigranular membrane with the cell membrane (arrow) in a sensitized mast cell treated with antigen for 60 s The structures of the membranes disappear at the site of contact × 112 000

Fig 7 Apposition and fusion of the membrane of a cavity containing changed granules and the perigranular membrane of an adjacent granule (arrows) Incubation time 60 s × 75 000

Fig 8 A sensitized mast cell treated with antigen for 90 s Many of the granules are swollen and have a decreased electron density Many of these granules are still retained in cavities in the mast cell with no visible communication with the extracellular milieu in the plane of sectioning × 8000

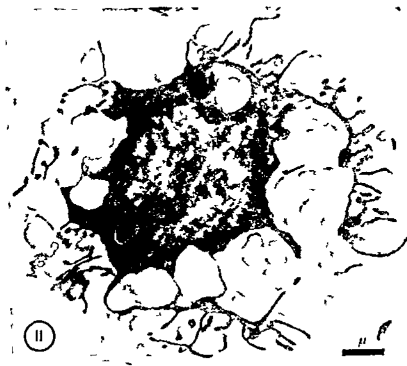


Fig 11 A mast cell treated with antigen for 10 min Almost all the granules are swollen and less electron dense Note that the cell still retains its shape through cytoplasmic strands connecting the periphery of the c ll with the nuclear region No structures except the granules appear to be changed ×11 000

Despite the fact that most of the granules in some mast cells were altered the cells still retained their original shape by means of peripheral cytoplasmic bridges connected to the nuclear region by cytoplasmic bands (Fig 11)

All mast cells examined at a certain time were not at the same stage of degranulation Even after the longest incubation time completely normal unchanged mast cells could be found although they were few in number

The cytoplasmic organelles of the sensitized mast cells did not show any morphological alterations during the antigen treatment

Lanthanum tracing

Sensitized mast cells not incubated with antigen showed a lanthanum precipitate on

Fig 9 Sens tized mast c ll treated ith antig n for 180 s The fusion process s seen to h ve pread tow rds the interior and laterally nto the mast cell and more granules are affected A thin d aphragm between ch nged granules a e een at arrows Granules lea ing the cell are also seen (double ar ow ×8500

Fig 10 An advanced tag of d granulation at 10 min ncubat on Some granules (\) have an electron densit betw n that of normal granules and that of released granules Many of the changed granules are also seen outs de the cell ×8500

the cell surface but the absence of a precipitate inside the mast cells showing that no lanthanum penetrated the cell membrane (Fig 12)

Sensitized mast cells treated with antigen (60 s) showed a completely different picture Besides the lanthanum precipitate adsorbed to the cell surface there was a precipitate lining the membranes of the cavities containing altered granules such granules had taken up the precipitate (Fig 13) Granules outside the cells were also deeply stained by lanthanum In the 60 s material the cavities were located near the cell surface and they were few in number (Fig 13) but after longer incubation times cavities could also be found penetrating more deeply into the mast cells (Fig 14)

A few swollen granules showed very little lanthanum precipitate (Fig 14 marked \) these granules were always located very close to the extracellular space either the cell surface or the cavities

Discussion

In the present investigation histamine was released when a mixed cell suspension from peritoneum and pleura of rats actively sensitized with egg albumin and pertussis vaccine (adjuvant) was incubated with antigen (5 μg egg albumin per ml) at 25° C Since mast cells are the only cells in this type of suspension containing releasable histamine (Fawcett 1954 Norn 1968) the histamine measured in the present study reflects the histamine release from mast cells

Light microscopic studies showed that before histamine release commenced the mast cell granules were stained dark blue with toluidine blue azure A solution However concomitantly with histamine release pink staining (metachromatic) granules were observed both in and around the mast cells The proportion of granules staining pink increased with increasing incubation time and histamine release

The initial ultrastructural changes in our material occurred after 60 s incubation and always started in the most peripherally situated granules lying immediately under the cell membrane These granules changed from homogeneous electron dense structures to granules with a swollen appearance a loose internal structure and a decreased electron density At this stage in the release process a contact and a fusion of the plasma membrane with the perigranular membrane could be seen at favourable planes of sectioning and very occasionally a thin structureless diaphragm could be seen over the fused region As the proces of degranulation continues this mem

Fig 12 Sensitized mast cell not treat d w th antigen shows a lanthanum precipitate adsorbed to the cell membrane (arrows) No prec p tate is seen inside the cell × 43 000

Fig 13 S nsitized mast cell treat d ith antigen for 60 s Lanthanum prec pitates are found at the cell surface l ning the membranes of the ca ities containing changed granules (ar ows) The changed granules ha e al o taken up the precipitate × 17 000

Fig 14 A longer time (180 s) of incubation with antigen The ca it es are now observed to penetrate deeper nto t e mast ell the membranes of these are l ned with the lanthanum precipitate (arrows) wh ch is also bound to t e changed granules either i de the ca ities or outside the cell Som granule are se n to b taking up the precipitate (\) × 8500

brane fusion process proceeds perigranular membranes fusing with the membranes of already formed granule cavities With longer incubation times more and more granules in the individual mast cells were changed the sequential process of mem brane fusions and granule changes (swelling decreased electron density) were seen to spread towards the interior of the mast cells and/or laterally In addition more and more mast cells were affected with time At the end of the incubation period complicated labyrinthic cavities containing changed granules were seen

As shown with the extracellular tracer substance lanthanum the changed granules situated in the newly formed cavities, were all in contact with the extracellular milieu even if such open communications with the extracellular space could not always be shown in individual sections

Sequential fusion of perigranular membranes by means of which the extracellular space penetrates deep into the cell interior and granules are extruded corresponds to exocytosis a phenomenon first described by Palade (1959) in the pancreatic acinar cell

Morphological changes analogous to those found in the present study have recently been described in mast cells exposed to the histamine releaser compound 48/80 (Rohlich *et al* 1971)

Uvnas has proposed that histamine release from mast cell granules is the result of a cation exchange between histamine ionically linked to the heparin protein com plex forming the granule matrix and cations in the extracellular fluid (Uvnas 1964 Uvnas and Thon 1966 Thon and Uvnas 1967) The histamine release induced by compound 48/80 was assumed to be a 2 step process an initial degranulation with expulsion of granules and a subsequent release of histamine from the granules ex posed to the cations in the extracellular medium Theoretically this mode of histamine release would require a 1 1 ratio between the amounts of histamine and granules leaving the cell However the histamine/granule (^{35}S or heparin) release ratio was found regularly to be above unity varying between 1 4—2 depending on the conc of compound 48/80 and the histamine release induced (Nosal Slorach and Uvnas 1970 Slorach 1971) Since granules expelled into isotonic salt solutions be

There could be 2 explanations for these observations Either all the histamine release without concomitant granule extrusion

There could be 2 explanations for these observations Either all the histamine was released from the granules by cation exchange but a proportion of the depleted granules was retained in the mast cells or 2 different release mechanisms one extra and one intracellular were operating

Bloom and Chakravarty (1970) stressed the point that mast cells under the in fluence of histamine releasers—antigen or compound 48/80—show structural changes in granule alterations not only at the periphery of the cells but also deep within the central parts of the cytoplasm They concluded it appears likely that histamine release can take place both intra and extracellularly They ascribe the altered appearance of cytoplasmic granules to an intracellular release possibly due to permeability changes in the perigranular membranes

Carlsson and Ritzen (1969) have for similar reasons proposed that 5 hydroxy tryptamine (5 HT) release from mast cells treated with compound 48/80 or antigen (sensitized cells) can occur intracellularly Using microfluorimetric and micro interferometric techniques they found namely a proportionally greater release of 5 HT than lowering of dry mass of individual mast cells Furthermore they reported the presence of granules which had lost their 5 HT fluorescence lying in cavities within the mast cells with no apparent connections with the extracellular space

The present and previous observations from our laboratory (*Röhlich et al 1971*) may explain the apparent discrepancies between the observations cited above and ours Using the tracer substances lanthanum and hemoglobin which do not penetrate intact membranes we have found that all changed granules retained in degranulated mast cells are located in cavities with open communication to the extracellular space Since the granules are totally depleted of their histamine and 5 HT when exposed to isotonic salt solutions the altered intracellular granules observed by Bloom and Haegermark (after compound 48/80) and by Bloom and Chakravarty (after antigen) as well as the non 5 HT fluorescent granules observed by Carlsson and Ritzen (after compound 48/80 and antigen) might in our view very well represent granules which have lost their histamine and 5 HT due to exposure to extracellular fluid pentrating into the cavities In this way all the histamine and 5 HT release—even that apparently taking place intracellularly—in degranulating mast cells might be explained as the result of one single mechanism an ion exchange at the granule sites between the amines and the cations in the extracellular fluid

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Differential Effects of Ethyl Alcohol on Retinal Functions

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Abstract

BERNHARD C G B KNAVE and H E PERSSON *Differential effects of ethyl alcohol on retinal functions* Acta physiol scand 1973 *88* 373—381

The action of ethyl alcohol on retinal fun tion was analyzed by comparing its effects on the conventional and low intensity ERG in the dark adapted intact sheep eye The results showed an increase of the *b* wave and a decrease of the *a* wave amplitude after small doses of alcohol (b a c ?0—40 mg %) The low intensity ERG *i e* the electroretinographic responses belo v *b* wave threshold has recently been shown to consist of a slow corneanegative receptor re sponse and tv o fast d c responses of opposite polarities from the inner nuclear layer The negative d c response is considered inhibitory in nature and the positive d c response ex citatory Administration of alcohol had no effect on the isolated receptor response but elicited an amplitude increase of the positive d c response The results from the low intensity ERG experiments suggest the mechanism behind the effects of alcohol on retinal funct ons to be a selective suppression of the negative d c response Hereby the controlling' function on the cells generating the positive d c response is suppressed thus releasing' the positive d c response and the *b* wave which has been shown to be built up by the positi e d c response The de crease of the *a* wave appears to be secondary to the increase of the *b* wave all the more as it was shown that alcohol had no effect on the receptor response At high blood alcohol con centrations the *a* wave as well as the *b* wave amplitude were found to diminish as an expres sion for a presumed general depression of the c n s

Ethyl alcohol has long been popularly regarded as a stimulant From a more biological point of view however there seems little doubt that ethyl alcohol (alcohol) is a primary depressant of the c n s and that the apparent stimulation is the result of a depression of certain inhibitory control mechanisms Such an effect for in stance was demonstrated on the retinal functions in the excised and opened eye of the frog (Bernhard and Skoglund 1941) and tortoise (Bernhard 194?) These authors recorded the ERG after local administration of alcohol and found the *a* wave to diminish and *b* wave to increase in amplitude This was interpreted as an effect of selective suppression of the negative component P III (in terms sug gested by Granit 1933) The ERG changes obtained by Bernhard and Skoglund were later confirmed in studies on the isolated retina of frog (Tomita Funaishi and Shino 1951 Forbes Burleigh and Nevland 19?5) and turtle (Forbes *et al* 19?5)

Bernhard and Skoglund also demonstrated a selective suppression by means of alcohol on the inhibition of the impulse discharge in the optic nerve during re

illumination after stimulation had ceased. This 'disinhibitory' effect on the discharge of the frog optic nerve was confirmed by MacNichol and Benolken (1956) who found a similar effect on the lateral inhibition in the functionally well-defined *Limulus* preparation.

Recently, as judged by results from experiments on cold-blooded vertebrates (Murakami and Kaneko 1966, Murakami and Sasaki 1968 a, b, Sillman, Ito and Tomita 1969) as well as on mammals (Hanitzsch and Trifonow 1968, Pautler, Murakami and Nosaki 1968, Knave, Møller and Persson 1972) it has been suggested that *P III* consists of a proximal part from the inner nuclear layer and a distal part from the receptor cells. In one of these works alcohol was applied to the isolated carp retina (Murakami and Sasaki 1968) and the effects obtained were interpreted as due to a selective suppression of the proximal *P III*.

The effects of alcohol on the mammalian ERG reported in earlier works were not quite consistent with those just described and to a certain extent they also were at variance with one another. In cat and rabbit only the *a* wave was left after the administration of high, lethal doses of alcohol (Praglin, Spurney and Potts 1955). On the other hand, after relatively small doses in the rabbit, an increase of the *b* wave amplitude was recorded (Straub 1960, Manfredini and Trimarchi 1968). In these works the *a* wave was not evaluated, since the stimulus intensities used were below *a* wave threshold.

In other studies again an increase of the *a* and *b* wave amplitudes were reported after small and moderate doses of alcohol intravitreally injected in the rabbit (Morita 1970) and intravenously in man (Jacobson *et al.* 1969). In another study on the ERG after peroral administration of a small dose of alcohol, the amplitude of the *b* wave was found to increase, while no change in the *a* wave could be observed (Ikeda 1963).

These seemingly controversial results prompted an approach with a new technique to the problem concerning the effects of alcohol on retinal functions. Recently a reinterpretation of the components of the mammalian ERG was presented (Knave, Møller and Persson 1972). This was based on longterm experiments in which summated retinal responses from the intact eye in sheep to light stimuli within an intensity range about 2.5 log units below the *b* wave threshold were recorded. This method has opened new possibilities for the evaluation of the effects of alcohol on retinal functions in experiments on the intact mammalian eye.

Methods

1[illegible] successful experiments were performed on sheep in the dark adapted state. The method used has recently been described in full (Knave *et al.* 1972).

Since the intensity amplitude curve of the *b* wave is known to have two saturation levels (see e.g. Arden, Granit and Ponte 1960, Knave *et al.* 1972) the stimulus intensity was always well above the lower of these levels. Duration of light stimulation was 0.1 s. Intervals between flashes were 1 min. The *a* wave was measured from the isoelectric line and the *b* wave from the trough of the *a* wave. When recording the summated ERG below *b* wave threshold, the duration of stimulus was one sec and the interval between flashes was 10 s. A 20 per cent solution (Ringer) of alcohol was administered either intravenously or via a ventriculotomy sno[illegible]

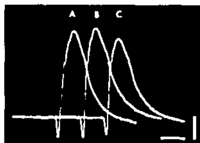


Fig 1 ERG of the dark adapted sheep eye before (A) and after (B and C) alcohol administration to the stomach The B and C records were taken at a blood alcohol concentration of 25 and 140 mg % respectively Stimulus intensity 5 0 log units above *b* wave threshold Stimulus duration 0 1 s Time calibration 0 1 s Amplitude calibration 200 μV

the stomach Capillary blood samples were taken and the ADH method (alcoholic dehydrogenase) was used for determining their concentration of alcohol[1]

Results

Effects of alcohol on single flash ERG at different blood concentrations

In studying the effects of alcohol on the ERG two reasons are in favour for choosing the stomach route for drug administration First this is the natural way Secondly the resorption is slow enough to allow a detailed study of the sequence of the effects As will be pointed out below however some effects could be demonstrated more

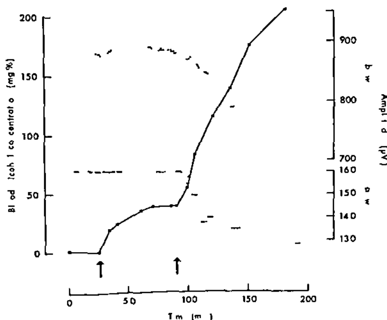


Fig 2 Effect of alcohol on *a* wave and *b* wave amplitudes of the dark adapted sheep eye 35 and 65 ml of a 20 per cent alcohol solution (Ringer) were administered to the stomach after 25 and 90 min respectively (arrows) Solid line between squares blood alcohol concentration in mg % Lower and upper arrays of filled circles denote *a* and *b* wave amplitudes respectively Stimulus intensity 5 0 log units above *b* wave threshold Stimulus duration 0 1 s

[1] The analysises were made at the Government Laboratory for Forensic Chemistry and a Department of Alcohol Research Karolinska institutet

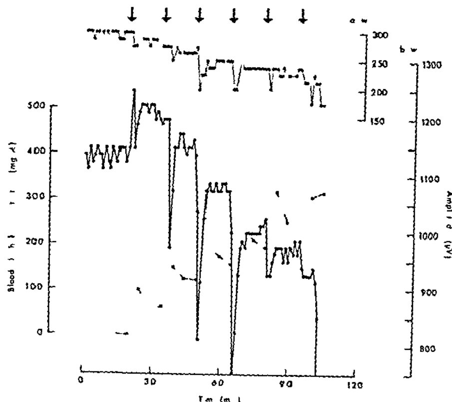


Fig 3 Effect of intravenously administered alcohol on *a* and *b* wave amplitudes of the dark adapted sheep eye 6 successive i v injections of 50 ml of a 20 per cent alcohol solution (Ringer) were given at 20 35 49 64 79 and 100 min respectively (arrows) Dashed lines blood alcohol concentration in mg % Dotted and solid lines denote *a* and *b* wave amplitudes respectively Stimulus intensity 4 7 log units above *b* wave threshold Stimulus duration 0 1 s

clearly after i v administration Thus typical results will be presented first from an experiment in which alcohol was given into the stomach and then from another experiment after i v injection of the drug

Fig 1 shows the configuration of the ERGs obtained before (A) and after administration of alcohol to the stomach (B and C) At low blood alcohol concentrations (b a c) the *b* wave amplitude was found to increase as illustrated by the record in Fig 1 B taken at a b a c of 25 mg % At high concentrations the *b* wave as well as the *a* wave amplitudes were found to diminish as seen in Fig 1 C the record being obtained at a b a c of 140 mg %

In Fig 2 the amplitudes of the *a* (lower array of filled circles) and the *b* wave (upper array of filled circles) as well as the b a c (squares solid line) are graphically illustrated from the same experiment After 25 and 90 min respectively (arrows) 35 and 65 ml of 20 per cent solution were given

After the first small dose of alcohol the b a c slowly increased to a plateau at 40 mg % and after the second dose there was a steep rise to high values (200 mg % at the end of the experiment)

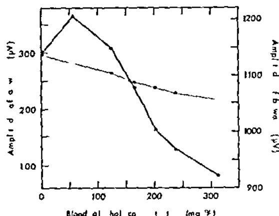


Fig 4 Effect of alcohol on *a* and *b* wave amplitudes of the dark adapted sheep eye The figures of this graph are obtained from the experiment illustrated in Fig 3 The means of the blood alcohol concentration 10 and 15 min after each injection are plotted against the mean amplitudes obtained during these periods

A temporary increase of the *b* wave amplitude was noted about five min after the first dose was given coinciding in time with the first slow increase of the b a c to a value of about 20 mg % (see also Fig 1 B) Between 70 and 90 min after administration *i e* when the b a c reached the plateau at 40 mg % the *b* wave amplitude returned to normal values After the second dose of alcohol there was a fast decrease of the amplitude of the *b* wave (see also Fig 1 C)

The *a* wave was found to be fairly constant up to the administration of the second dose of alcohol after which the *a* wave amplitude also diminished As a matter of fact, a decrease in amplitude was vaguely indicated already before the second dose during the time when the b a c reached its plateau at 40 mg %

This suspected decrease of the *a* wave amplitude at low b a c was manifested after giving the alcohol intravenously In Fig 3 the amplitudes of the *a* wave (dotted line) and *b* wave (solid line) as well as the b a c values (dashed line) are shown after six successive i v injections of 50 ml of a 20 per cent alcohol solution at 20 35 49 64 79 and 100 min respectively (arrows) Determination of the b a c was made 5 10 and 15 min after each injection (except for the last one when blood samples were taken after 5 and 10 min only)

After each injection the b a c increased the value obtained 5 min after each injection being higher than the values obtained after 10 and 15 min Rapid transient changes in the ERG amplitudes also occurred after each injection Constant values were not obtained until after about 5 min The initial transient changes were most probably due to a redistribution of the injected alcohol (see Ritchie 1970) Since the central nervous tissue has a rich blood supply and a high coefficient of affinity for alcohol the effects on c n s are especially rapid after i v administration However the alcohol is soon redistributed to other tissues and not until this redistribution is finished can constant values be expected The transient effects immediately after the injections will not be dealt with further since we do not know the actual concentrations of alcohol either in the blood or in the retina during these

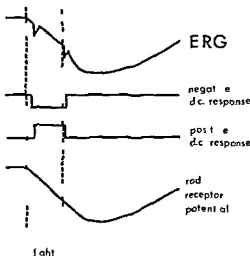


Fig 5 Schematic representation of ERG components below *b* wave threshold of the dark adapted sheep eye Upper diagram depicts a typical low intensity ERG which is assumed to be built up of a receptor response (lower diagram) and a negative and a positive d c response (middle diagrams)

It is more appropriate to evaluate the effects 10—15 min after each injection because at that time the b a c has reached constant levels

As can be seen in Fig 3 there was an enhancement of the *b* wave amplitude after the injection of the first dose of alcohol Increased amplitudes were still obtained after the second dose although their magnitude was now less than after the first dose The amplitudes diminished below the original level after the third dose and after the fourth fifth and sixth doses successively decreasing amplitudes were recorded The *a* wave amplitude did not increase initially instead there was a stepwise decrease with every injection

These changes in ERG amplitudes are depicted in the graph of Fig 4 Here the means (six individual values) of the b a c 10 and 15 min after injection are plotted against the mean amplitude obtained during these periods The decrease in *a* wave amplitude (dotted curve) was found to be almost linear with increasing concentrations of alcohol The solid line shows the initial increase and subsequent decrease of the *b* wave amplitude

Effect of alcohol on the summated ERG below b wave threshold

Recently a reinterpretation of the components of the ERG was presented (Knave *et al* 1972) This analysis was based mainly on averaged responses below *b* wave threshold of the dark adapted intact sheep eye At these low stimulus intensities it was possible to separate three components which were assumed to represent a rod receptor potential and a negative and a positive d c response generated in the inner nuclear layer Such a low intensity ERG is schematically depicted in Fig 5 (upper diagram) as well as its components i e the rod receptor potential (lower diagram) and the d c responses (middle diagrams)

Furthermore the negative d c response was tentatively interpreted to have an inhibitory effect on the activity giving rise to the positive d c response It was suggested that the negative d c response represented the proximal P III This is of special interest in as much as alcohol has been claimed to selectively suppress the



Fig 6 (left) Effect of alcohol on the ERG below b wave threshold in the dark adapted sheep eye The slow corneanegative potential represents the isolated receptor response elicited just above its threshold with a stimulus intensity 2 4 log units below b wave threshold 100 summated responses are shown before (A) and 10 min after i v administration of 50 ml of a 20 per cent alcohol solution (B) The blood alcohol concentration determined immediately before and after the latter ERG summation (record B) were 60 and 70 mg % respectively 1 s light stimulus marked in the lowest record Time calibration 2 s Amplitude calibration 1 μV

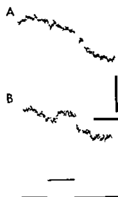


Fig 7 (right) Effect of alcohol on the ERG below b wave threshold in the dark adapted sheep eye The records are taken at a stimulus intensity of 1 8 log units below b wave threshold At this stimulus intensity the summated response has a configuration similar to that of the top record in Fig 5 100 summated responses are shown before (A) and 10 min after i v administration of 50 ml of a 20 per cent alcohol solution (B) The blood alcohol concentration determined immediately before and after the latter ERG summation (record B) were 50 and 60 mg % respectively One sec light stimulus marked in the lowest record Time calibration 1 s Amplitude calibration 10 μV

proximal P III (Murakami and Sasaki 1968 b) Thus there are reasons to expect an effect of alcohol on the d c components of the low intensity ERG

The experiments on the low intensity ERG were designed to study the effects on the receptor response isolated just above its threshold (Fig 6) as well as on the d c responses at somewhat higher stimulus intensities (Fig 7) The averaged ERGs were elicited by one second flashes with an intensity 2 4 and 1 8 log units below the b wave threshold (Fig 6 and 7 respectively) before (upper records) and 10 min after i v administration of 50 ml of a 20 per cent alcohol solution (lower records) B a c determined immediately before and after the latter averagings varied between 50 and 70 mg % i e the concentration at which maximal b wave amplitudes were obtained after i v injection (see Fig 4)

As can be seen in Fig 6 the shape and amplitude of the isolated receptor response were of the same order of magnitude before and after alcohol administration At somewhat higher stimulus intensities however the electroretinographic response changed in configuration after alcohol (Fig 7) A positive plateau developed between the two negative notches on the falling phase of the receptor response indicating an increase of the positive d c response (cf Fig 5)

Discussion

The result of the present studies on sheep shows that small doses of alcohol are followed by an increase of the *b* wave, as was first shown in experiments on cold blooded animals (Bernhard and Skoglund 1941, Bernhard 1942). The reduction of the *a* wave observed is also in accordance with the earlier results on cold blooded animals but not with the results reported in man (Jacobson *et al.* 1969) and rabbit (Morita 1970), where the *a* wave was found to increase after small doses of alcohol.

In the recently performed analysis of the mammalian ERG by Knave *et al.* (1972) three main components have been separated (see above). According to the present study on the effects of alcohol on the low intensity ERG there was no effect on the isolated receptor response but a clear amplitude increase of the positive d.c. response at somewhat higher stimulus intensities (see Fig. 6—7). In the above mentioned work it was concluded that the negative d.c. response is inhibitory and the positive one excitatory in nature. The authors also suggested that the negative d.c. response exerts an inhibitory influence on the functions signalled by the positive one. *With this theory in mind it is tempting to suggest that the primary effect of* alcohol on the ERG is a selective suppression of the negative d.c. response. By such a mechanism the controlling function on the positive d.c. response is suppressed, thus releasing the positive d.c. response. It should also be pointed out that the negative d.c. response has been suggested to correspond to the so called proximal P III (see introduction). Thus the finding that alcohol primarily suppresses the [illegible]imal P III (Murakami and Sasaki 1968 b) is supported by the results of the [illegible]sent study.

The results indicating that the primary effect of alcohol is a selective suppression of the negative d.c. response may also serve as an indirect confirmation of the alcohol effects recorded by Negishi and Svaetichin (1966). These authors found that alcohol in low concentration suppressed the function of cells producing the S potential whereas the photoreceptor cells, spike producing neurons and synaptic transmission had a greater resistance to the drug. In the reinterpretation of the ERG components mentioned above suggestive evidence was presented for the conclusion that the negative d.c. response was generated in the inner nuclear layer, probably by the horizontal cells, which have been shown to generate the S potentials (Kaneko 1970).

The complementary results on the effects of alcohol on the low intensity ERG may offer an explanation of the increase of the *b* wave and decrease of the *a* wave amplitude at low concentrations of alcohol in the blood. As mentioned above the most striking effect of alcohol is the amplitude increase of the positive d.c. response (see Figs. 5—7) which has been shown to build up the *b* wave (Knave *et al.* 1972). Since increase of the positive d.c. response appears to represent a release of the activity of cells in the inner nuclear layer from an inhibitory action exerted from the horizontal cells, the increase of the *b* wave appears to be a sign of this disinhibitory effect. The decrease of the *a* wave appears to be secondary to the *b* wave

increase all the more as it was shown in the present study that administration of alcohol had no effect on the isolated receptor potential (see Fig 6)

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Differential Effect of Graded Contraction of Middle Ear Muscles on the Sound Transmission of the Ear

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Abstract

TEIG E *Differential effect of graded contraction of middle ear muscles on the sound transmission of the ear* Acta physiol scand 1973 *88* 382—391

The middle ear muscles in cats have been brought to different degrees of contraction by electric stimulation through electrodes placed in the proper nuclei in the brain stem The resulting change in the amplitude of the cochlear microphonic potentials produced by pure tones from 250—7000 Hz has been used to determine the change in the sound transmission of the ear Later the muscle tension produced by the same stimulus strengths have been determined myographically The main effect of weak contractions involving only a relatively small number of motor units was more selective than the effect of stronger muscle contractions Contractions of the stapedius muscle involving only up to 10—15 per cent of the maximal contraction force reduced the microphonics below 2000 Hz with most marked effect on the lowest frequencies tested Stronger tensions produced an additional modest reduction equally pronounced for all sound frequencies The most marked effect of contractions of the tensor tympani amounting to 10—15 per cent of the maximal contraction force was a reduction of microphonics of frequencies below 750 Hz while higher frequencies could be either slightly enhanced or reduced Stronger contractions had a less selective effect with respect to various frequencies and gave less reduction of the sound transmission per gram tension

In most studies where different degrees of middle ear muscle tension have been correlated with the corresponding effect upon the sound transmission of the middle ear relatively strong tensions have been used and a certain degree of reduction of the sound transmission has been the common finding (Wever and Bray 1937 1942 Neergaard *et al* 1963 Cancura 1970 giving support to the notion that the muscles have mainly a protective function against excessive sound (Wever and Lawrence 1954) However in experiments in which the muscles have been brought to light contraction either through reflex activation (Wever and Vernon 1956 Price 1963 a b) or by other means (Starr 1969) enhancement of certain frequencies has been the common finding suggesting that weak contraction of the middle ear muscles has a different and more selective influence on the sound transmission than strong contraction of the muscles

The pattern of this possible selective action is not clear. During spontaneous contractions of the middle ear muscles in the guinea pig Wiggers (1937) found a reduction in the transmission of frequencies below 1000 Hz and a slight enhancement of frequencies from 1000 Hz up to 2500 Hz, while Møller (1965) in both cats and rabbits found that contraction of either or both middle ear muscles reduced frequencies below 2000 Hz leaving higher frequencies practically unaffected.

In apparent contrast to these findings are the results of Wever and Vernon (1955) and Price (1963 b) who used 450 Hz as a test tone in cats and rabbits and found enhancement of the transmission of this frequency during weak reflex activation of the middle ear muscles.

The main difficulty in correlating the reported data is that none of the investigations included measurements of the muscle tensions being exerted. Since the tensions of individual motor units of the middle ear muscle are now known (Teig 1972 b) and since ordinarily the weakest motor units in a muscle are activated first and used most frequently (Henneman and Olson 1965) we can estimate the tensions most often produced by the muscles under physiological conditions.

The purpose of the present investigation was to test if the effect of different degrees of weak contractions involving only a relatively small number of motor units was more selective and different from the effect of stronger muscle tensions. It was found that the main effect of weak to moderate contraction of the muscles was a reduction of the transmission of tones below certain frequencies (2000 Hz and 750 Hz for the stapedius and the tensor tympani, respectively) while the transmission of higher frequencies could be moderately enhanced or reduced. Additional contraction force resulted in a further moderate reduction of the transmission of all frequencies.

Methods

Operative procedure. Adult cats (1.9—3.9 kg) were anesthetized with sodium pentobarbital (30 mg/kg), tracheotomized and fixed in a headholder. A retroauricular incision through the skin and the platysma exposed the posterolateral parts of the left mastoid bulla, and after cutting through the cartilagenous part of the ear canal, good access to the mastoid bulla was obtained. This was drilled open exposing the round window, and after removal of a part of the septum between the middle ear and the left mastoid bulla, access to the belly of the tensor tympani muscle was obtained. The canal of the facial nerve was drilled open from the posterolateral part of the bulla to the level of the stapedius muscle. A small part of the stapedius muscle was exposed to allow electromyographic recording. Bipolar 0.5 mm insulated silver electrodes were then placed on the surface of the tensor tympani and the stapedius muscles. A 1 mm ball point silver electrode was put on the rim of the round window to record the cochlear microphonic potentials, while a similar indifferent electrode was placed on the surface of the parotid gland which was exposed during the operation. The septum between the middle ear cavity and the mastoid bulla, and later the mastoid bulla itself, were then reconstructed by dental acrylic, leaving the electrodes secured in place. The posterior part of the skull was opened and the posterior part of the cerebellum removed by suction, exposing the floor of the fourth ventricle. Using the obex as a reference, stimulating electrodes held in a stereotactic manipulator could be placed in the motor nucleus of the trigeminal nerve and in the facial nucleus (Teig 1972 b).

Stimulation and recording technique. Since it has proved difficult to reproduce acoustically elicited middle ear muscle contractions in anesthetized cats (Møller 1965), electric stimulation of the proper motor nuclei in the brain stem was used to activate the muscles. Bipolar insulated tungsten electrodes (tip diameter about 10 μm, 1.5—2.0 mm apart) were used as stimulation electrodes and inserted in the facial nucleus or in the motor nucleus of the trigeminal ne

depending on which muscle being tested. When a preliminary localization of the appropriate nucleus has been obtained stereotactically, negative square pulses of 0.2 ms duration and an amplitude sufficient to elicite muscle action potentials in one of the middle ear muscles were applied. Checks were made that only one muscle was excited.

Usually the electrodes had to be moved several times to find a location close enough to the motor nucleus of one muscle to avoid simultaneous stimulation of the other muscle when the stimulus strength was increased. Pure tones from 200 to 7000 Hz were applied to the operated ear through an earphone type Telephonics TDH 39 attached to a rubber tube (30 mm long—diameter 12 mm). To ensure a closed cavity the rubber tube was glued to the temporal bone around the ear canal with dental cement. When calibrated in a 2 cm^3 coupler with a Bruel and Kjaer condenser microphone type 4134 pure tones of 60—90 dB would give a cochlear microphonic potential of about 400 μV. Input/output curves of applied sound/microphonic potentials showed that this was within the linear response when plotted out in dB. The phenomenon of overloading (Stevens and Davis 1938) did thus not interfere with the interpretation of the results.

The electromyograms were amplified, displayed on separate beams on the oscilloscope and served as control to show which muscle was tested. With the depth of anesthesia employed no reflex contraction of the middle ear muscles took place, judged from the electromyographic response. Trains of 0.2 ms negative square pulses with a frequency of 100 Hz and duration of 150 ms, which was known to give tetanic fusion of the middle ear muscles (Teig 1972 a), were then applied. This relatively short stimulation period was employed to avoid muscular fatigue even if the period was not long enough for the muscles to reach the tension plateau (Teig 1972 b). The stimulus intensity was varied in small steps and the amplitude of the electromyogram and the effect on the transmission of a 500 Hz tone were used as a control of the stability of the preparation. Series and experiments during which these control records changed were discarded. In experiments where the motor nuclei of the muscles were found only after prolonged manipulation they had a tendency of being reduced, probably due to local damage of the brainstem.

To rule out the possibility that accidental stimulation of the olivocochlear bundle should influence the results (Fex 1962), the tendons of the middle ear muscles were cut in two separate experiments. Stimulation of the motor nuclei then did not give any change in the microphonic potentials.

At the end of each experiment the bulla and the middle ear were widely exposed and the tensor tympani and the stapedius muscles were attached to an electromechanical transducer. This part of the procedure has been described previously (Teig 1972 b). The optimal length of the muscles was determined by applying strong single pulses (0.2 ms duration) and observing the size of the resulting twitch (Teig 1972 a). The tetanic contraction of the muscles at optimal length was then recorded at the stimulus intensities used during the microphonic experiments. The tension was maximal approximately 10 ms after the last stimulus pulse and the tetanic tension as well as the effect on the microphonic potentials were therefore both measured at this point.

Results

The effect upon the sound transmission of small tensions of either of the two muscles was dependent both on the sound frequency tested and upon the particular tension being used. In preliminary experiments single muscle twitches were used to modulate the microphonic potentials. Fig. 1 A shows the transient change of the microphonic potentials of a 500 Hz tone caused by a tensor tympani muscle twitch and Fig. 1 B shows the effect of the same twitch upon the 2000 Hz microphonic potentials. The mechanical record of the twitch is shown in the lower row for comparison. Muscle contraction caused a reduction of the microphonic potentials of the test tones. This reduction often matched the time course of the contractions but could also particularly with stronger contractions have a different shape. The matching was best at lower frequencies (500 Hz). When testing with higher sound frequencies (2000 Hz) DC components made the recordings difficult to interpret. These changes were

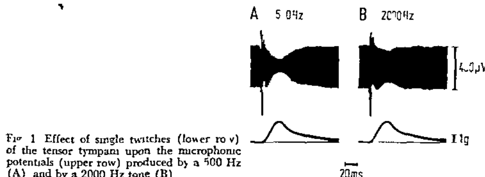


Fig 1 Effect of single twitches (lower row) of the tensor tympani upon the microphonic potentials (upper row) produced by a 500 Hz (A) and by a 2000 Hz tone (B)

similar to those described by Galambos and Rupert (1959) Because of the distorted shape of the recordings at higher test frequencies it was decided to used tetanic stimulation The smoother tetanic contraction curve reduced these disturbances Measurements at the end of a tetanic contraction further avoided the initial acoustic transients caused by abrupt tension changes not only in the ossicular chain but al o in the ear drum and in the supporting ligaments

Fig 2 exemplifies some of the effects of tetanic contraction of the tensor tympani muscle upon the microphonic potentials recorded The upper row shows the myographic recording of a submaximal muscle contraction (A) with peak tension of 1 2 g and the effect of this contraction upon the 500 Hz (B) 750 Hz (C) and 1000 Hz (D) microphonic potentials The corresponding electromyograms (EMG s) of the tensor tympani and the stapedius muscles are shown under each recording of the microphonic potentials Only the tensor tympani muscle was active The lower row (E F G and H) shows the corresponding records of a stronger contraction with peak tension of 4 5 g A light contraction reduced the transmission of the 500 Hz tone (Fig 2 B) while at the same time enhancing the transmission of the 750 Hz tone (Fig 2 C)

This enhancement of the transmission of certain frequencies was regularly seen to follow light contraction of both the stapedius and the tensor tympani The effect of a relatively strong muscle contraction (Fig 2 F H) usually reached its maximum be fore the muscle contraction itself reached its maximum as judged by the myographic recording (Fig 2 E) This apparent saturation of the effect of the muscle contrac tion was seen with both stapedius and tensor tympani contractions

The effect upon the sound transmission of stapedius contractions giving tensions between 400 mg and 6 5 g was studied in seven experiments Selected frequencies from 250 Hz to 6000 or 7000 Hz were used as test tones The reduction of the micro-phonic potentials was most pronounced for the lower frequencies particularly at small tensions The experiment shown in Fig 3 A and 4 A exemplifies the general conclusion on the stapedius muscle In Fig 3 A the change in microphonic potential amplitude produced by different muscle tensions is given in dB with the initial 400 µV microphonic potentials as reference level Increasing muscle tension up to

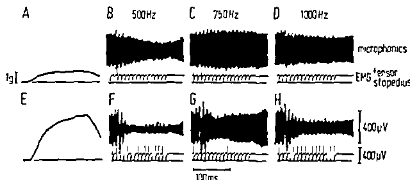


Fig. 2. Upper row shows a tetanic contraction (A) of the tensor tympani with a peak tension of 1.2 g and the effect of this contraction upon the microphonic potentials produced by a 500 Hz (B), a 750 Hz (C) and a 1000 Hz (D) tone. The two tracings below the microphonic potential trace are from above the electromyograms of the tensor tympani and the stapedius muscles respectively. Lower row (E, F, G and H) gives similar data but with stronger tetanic tension (peak tension of 4.5 g).

2.10 g led to a decrease in the microphonic potential amplitude at frequencies below 3000 Hz. The effect was most pronounced for the lowest frequency tested, 250 Hz. Small tensions up to 1.27 g gave improvement of the transmission as seen by increased microphonic potentials up to 0.5 dB in the 3000—4000 Hz range. Additional increase in the muscle tension gave a reduction of the microphonic potentials at all frequencies.

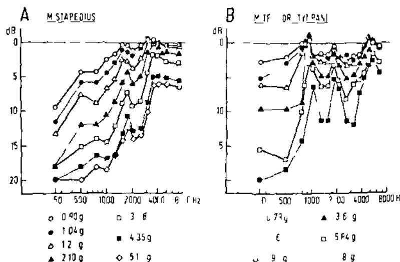


Fig. 3. Change in the microphonic potential amplitude at different test frequencies with the initial 400 µV responses as the reference level produced by the muscle tensions indicated by the symbols under the graph. A. Stapedius muscle. B. Tensor tympani muscle.

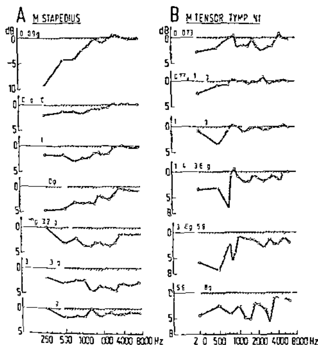


Fig 4 Incremental effect upon the cochlear microphonic potentials at different frequencies produced by stepwise changes in muscle tension as indicated above each diagram A Stapedius muscle B Tensor tympani muscle

Fig 4 A which is from the same experiment as Fig 3 A shows the incremental effect upon the transmission of different sound frequencies of stepwise increased tensions The upper diagram shows the effect of an increase in muscle tension from zero to 0 9 g the next graph the effect of increasing the muscle tension from 0 9 g to 1 04 g The subsequent graphs similarly show the effect of increases in the muscle tension given by the numbers above each diagram In a previous inactive muscle an increase in muscle tension to about 1 0—1 5 g mainly affected the frequencies below 2000 Hz At the lowest frequencies the sound reduction caused by such a tension increase could be up to 12—15 dB When a similar degree of tension was added to a previous tension of 2—3 g this gave an additional reduction in the sound transmission of 2—3 dB only and had an almost equal effect over the whole frequency range

The effect of tensions in the tensor tympani between 650 mg and 12 6 g (6 expts) upon the sound transmission of different frequencies is exemplified by the experiment shown in Fig 3 B and 4 B In Fig 3 B the change in transmission produced by various tensions in the tensor tympani is given in dB with the initial 400 μV microphonic potentials as reference level Fig 4 B which is from the same experiment shows the net effects for the six step increases of muscle tension as indicated above each graph Tensions up to 5 84 g gave a reduction in the sound transmission of most frequencies below 4000 Hz The reduction was most marked for frequencies below 750 Hz This effect was accompanied by peaks of absolutely or relatively in

creased transmission in certain frequency ranges In the 6 ears tested 3 peaks of absolutely or relatively increased transmission were found, one in the 750—1200 Hz range a second in the 1500—2500 Hz range and a third in the 4000—6000 Hz range In each ear these peaks of absolutely or relatively increased transmission had a surprisingly narrow range such as shown in Fig 3 B

As in the stapedius muscle the effect of increasing the tension of the tensor tympani muscle was dependent upon the previous tension in the muscle In an inactive muscle an increase in muscle tension to 5—6 g had its strongest effect upon frequencies below 750 Hz giving maximal sound reduction of more than 15 dB A similar tension added to a tension of 5 84 g gave nearly equal effect upon all frequencies up to 4000 Hz amounting to 3—5 dB only (Fig 4 B)

The effect of simultaneous contraction of both muscles was studied in four experiments Since the relative activity of the two muscles under physiological conditions is not known the experiments were limited to test if the overall effect of the two muscles was qualitatively the same as that of each muscle alone The effect of simultaneous contraction of the two muscles was similar to that produced by each muscle alone and there was no simple summation of the effect of the two muscles

Discussion

Effect of contraction of the tensor tympani muscle

Except for the very weakest tension light to moderate contraction of the tensor tympani had a rather selective depressive effect upon the transmission of frequencies below 750 Hz while the additional effect of stronger contraction was a reduction of the transmission of all frequencies The effect of a muscle tension of 2—3 g closely resembled that found by Wever and Vernon (1955) during tensor tympani contraction elicited by acoustic stimulation of the contralateral ear 2 dB above reflex threshold (stapedius tendon cut) The difference between the present findings and the relative insignificance of the tensor tympani in the reduction of cochlear microphonic potentials in awake versus anesthetized cats (acoustic reflex inactive) reported by Simmons (1959) is hard to account for Since frequencies below 500 Hz were not tested by Simmons and probably weak tensor tympani contractions were elicited a possible depression of transmission of the lower frequencies might have escaped notice In the present study stronger tensions always produced reduction of the transmission of low frequency sound This naturally also applied to the effect of tetanic versus twitch contractions This is in contrast to Starr s (1969) observation that single shocks produced stronger reductions of low frequency sound than tetanic stimulation did Since the present experimental arrangement resembled that used by Starr (1969) there is no obvious explanation for this apparent discrepancy

The tetanic tensions or sizes of limb muscle motor units are inversely related to the reflex excitability of their motoneurones (Henneman and Olson 1965) Furthermore in the tensor tympani low voltage motor units with low firing frequency are activated before high voltage motor units with high firing frequency (Okamoto

Sato and Kirikae 1964) It therefore seems probable that the slow twitch motor units (Teig 1972 b) are activated first during physiological activation of the tensor tympani muscle These motor units had an average twitch tension of 26 4 mg Each tensor tympani muscle was estimated to consist of an average of 40 such slow twitch motor units Assuming a tetanus/twitch ratio of 4 these motor units together when maximally activated should be capable of producing a tension of approximately 4 g This is the tension range within which the relatively selective reduction of low frequency tones (below 750 Hz) was found to take place

The less selective influence upon the sound transmission when the muscle tension exceeded 5—6 g and the relatively modest additional damping effect of such contractions suggest that reduction of low frequency transmission is only one aspect of the function of this muscle It should be kept in mind that the 5—6 g tension which gives the most selective high pass filter action represents only about 10 per cent of the maximal contraction force of this muscle (Teig 1972 a)

Effect of contraction of the stapedius muscle

Weak to moderate contraction of the stapedius muscle produced a change in the transmission of the middle ear similar to that found during stapedius muscle contraction in cat (tensor tympani anesthetized) elicited by acoustic stimulation of the contralateral ear (Møller 1965) suggesting that in the latter experiments the contraction force of the muscle was about 1—1 5 g The effect of the weakest contractions used during the present investigation (400 mg) was similar to but more pronouned than that obtained by Wever and Vernon (1955) using acoustic stimulation of the contralateral ear 2 dB above reflex threshold and having the tensor tympani cut The effect on the transmission pattern was also similar to that found in man during electric stimulation of the external ear canal (Pichler and Bornschein 1957)

The twitch tension of the stapedius motor units ranges between 11 and 168 mg (Teig 1972 b) Assuming a tetanus/twitch ratio of 4 the tetanic tension of individual motor units upon maximal stimulation should range between 40 and 750 mg The supposedly slow muscle fibres of the stapedius which had a smaller diameter than the fast ones (Teig and Dahl 1972) may constitute even weaker motor units than the fast twitch type Since the smallest motor units in a muscle are most numerous (Henneman and Olson 1965 Teig 1972 b) the stapedius muscle is probably capable of an extremely fine gradation of the effect upon the sound transmission of frequencies below 1500 Hz Stronger contractions of the stapedius muscle produced a change in the sound transmission similar to that found in artificial tension studies on temporal bone preparations in cat (Wever and Bray 1942) and in man (Neergaard *et al* 1964) The effect upon the sound transmission of tension in excess of 2 g was almost similar for all frequencies Since 2 g represent only about 15 per cent of the total contraction force of the stapedius muscle in the cat (Teig 1972 a) these data suggest an additional function for this muscle to that of reducing the transmission of low frequency sound The reductions of transmission for a given amount of tension were much greater for the stapedius muscle than for the tensor

tympani an observation which was also made by Wever and Bray (1937, 1942) From the description of the middle ear as a mechanical transformer (Wever and Lawrence 1954) it appears that the distance from the rotational axis of the ossicular chain to the insertion of the stapedius muscle is considerably longer than the distance to the insertion of the tensor tympani The lever arm of the stapedius muscle is thus longer than the lever arm of the tensor tympani This offers one explanation for the difference in effectiveness per gram force of the two muscles

Possible physiological significance of middle ear muscle contraction

Since low frequency tones mask high frequency tones far more effectively than vice versa the differential suppression of low frequency tones as pointed out by Stevens and Davis (1938) improves hearing for faint high frequency tones in the presence of loud low frequency tones The present finding that the tensor tympani muscle upon mild to moderate contraction had the strongest damping effect upon sound below about 750 Hz might give this muscle a role for selective removal of masking when the animal is directing its awareness against weak sounds of higher frequencies The importance of the stapedius muscle as a high pass filter was emphasized by Liden Nordlund and Hawkins (1964) who found stapedectomized patients with the stapedius tendon cut to have a poorer discrimination in noise than stapedectomized patients with comparable hearing and an intact stapedius muscle function

Even if peaks of absolutely or relatively increased transmission were a frequent finding in the present study these peaks had almost the same location during different degrees of muscle tension. The continual shift in resonant loci believed to take place during small changes in muscle tension (Simmons 1964) could not be confirmed

Enhancement of low frequency sound such as the 450 Hz frequency found in a certain proportion of cats (Wever and Vernon 1956) and rabbits (Price 1963 b) during acoustically elicited contraction of both middle ear muscles was not observed with the muscle tensions used during the present study It is possible that this occurs in some animals during very weak contractions of the muscles as a by product of the change in tension as suggested by Stevens and Davis *(1938)* The possibility that the two muscles should have a quite different effect when operating together than when operating alone seems unlikely both from the present experiments and from previous work (Moller 1965 Wever and Vernon 1955 Neergaard *et al* 1964)

The relatively moderate reduction in the transmission of all frequencies which developed when the tension of the middle ear muscles exceeded about 10—15 per cent of the total contraction force is surprising However this sound reduction could be a less important effect of the muscle tension necessary to give sufficient contact pressure between the auditory ossicles at higher sound levels (Bekesy 1960)

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Acta physiol. scand 1973 88 392—400
From the Institute of Physiology University of Oslo Norway

Appearance of Red Cells in Peripheral Lymph during Radiation-Induced Thrombocytopenia

By

I AURSNES

Received 16 October 1972

Abstract

AURSNES I *Appearance of red cells in peripheral lymph during radiation induced thrombocytopenia* Acta physiol. scand 1973 88 392—400

Some effects of marked thrombocytopenia have been studied in rabbits exposed to whole body irradiation with the ears shielded. In most animals marked thrombocytopenia was present 9—13 days after the irradiation. Lymph samples were obtained by draining a lymph vessel at the base of the ear In 7 out of 11 rabbits a marked rise in the content of red cells in ear lymph occurred during thrombocytopenia. In the 4 other irradiated rabbits which developed the same degree of thrombocytopenia, no such red cell increase in the ear lymph occurred The reason for this is not known Platelet production may have been resumed at an earlier stage in these 4 animals. The first few platelets produced at the end of the thrombocytopenic period were apparently consumed in repairing the endothelium This notion is supported by the fact that in some of the rabbits of the former group the erythrocyte-containing lymph was normalized one day before the number of circulating platelets started to rise. All rabbits showed a steady fall in body weight after irradiation In the rabbits with red cell increase in lymph however a moderate whole body weight gain could be observed at the time with marked thrombocytopenia. This suggests the existence of a slight degree of oedema during thrombocytopenia in these animals

It is generally accepted that there exists a vascular defect in individuals with thrombocytopenia The nature and extent of this defect is however not clear A definite sign of a generalized vessel wall defect in patients with a low number of circulatory blood platelets is petechial bleedings (Humble 1949 Kramar 1961) In thrombocytopenic animals the appearance of an increased number of red blood cells can be observed in thoracic duct lymph (Hjort *et al* 1959 Woods *et al* 1953) The escape of red blood cells from the blood vessels is thus apparently a key event in the affected vessels of thrombocytopenic individuals But how wide spread is this defect, and exactly how are the blood platelets involved The red cells appearing in the thoracic duct have been believed to originate from generalized red cell extravasation through anatomically intact capillaries in the tissues (Ross *et al* 1957 Van Horn and Johnson 1966) However other possible mechanisms behind the red cell appearance in the thoracic duct lymph have not been excluded in previous

studies The red cells could have entered the lymph in lymph nodes or they could originate only from capillaries in special tissues with fenestrated vascular endo thelium as e g the liver They could al o have gained access to lymph through lympho venous anastomosis Finally they could reflect erythrocyte extravasation at sites where capillaries had been subject to trauma

The intention with the present investigation has been to study further the vascular wall defect in thrombocytopenic animals as it is reflected in the leakage of red cells to the lymph The experimental situation has been simplified in that one has studied the appearance of red cells not in thoracic duct lymph but in peripheral lymph that is lymph withdrawn before it has passed through any lymph node This lymph was collected from rabbit ears an area which can easily be shielded during irradiation and where tissue traumatization can usually be avoided

A short report of some of these findings has been given earlier (Aursnes and Waaler 1972)

Materials and Methods

Rabb ts of a local albino strain and weighing 2 3—3 5 kg were used They were kept on a standardized pellet food and were given water *ad lib tum* during the experimental period We hing of animals and blood sampling (from ear veins) were performed at the same time each morning

Whole body irradiat on was performed according to principles described by Cronkite (1964) with gamma rays from a 7 MeV therapy linear accelerator at a distance of 160 cm This arrangement gave about 75 rads per min in the median plane of the body The rabbits w e e kept in restraining cages during irradiation and the dose as divided with one half to each side of the animals

Midline doses given ranged from 600 to 735 rads and were usually about 660 rads The doses were calculated from measurements made with an ionization chamber at the body surface The ears were shielded with 6 cm lead which reduces the local irradiation by 95 %

Platelet counts in blood were done by a separation method with electronic particle coun ing as pre iously described (Aursnes 1971) Counts below 100 000 per μl w e e corrected for the red cell admixture to the platelet su pension This adm ture was also evaluated by electronic counting in the same sample (Foss *et al* 1960) The reproduc b l ty of platelet counts in dupli cate blood samples was remarkably good In the last 25 measurements with blood platelet values below 100 000 μl blood the average difference between parallel samples was 2600/μl At very low blood platelet values the accuracy of the method was egularly checked by careful direct platelet counting with phase contrast microscopy (Brecher and Cronk t 1950)

Leucocytes in blood were counted b electronic particle counting (Celloscope 101 L nson Instrument Lars Ljungberg & Co Stockholm)

Hematocrit values of blood were obtained with a microhematocrit centrifuge

Exper mental arrangements

Lymph vessel cannulations were performed in l ght p ntobarbitone (Nembutal®) ane thesia (20—40 mg/kg) The thrombocytopenic an mals appeared to be particularly sensitive to pento barb tone and were given moderate doses (20—30 mg/kg) for their anesthesia A small qu nti ty of Patent Blue d ssol ed in saline (about 0 1 ml) was injected subcutaneously into the centre of the rabbit s ear just anterior to the median vessels After a short period of t me (1—2 min) a lymph vessel could be seen in the proximal part of the ear immediately belo the epidermis Under a dissection microscope a 5 mm long inci on was made through the skin and the lymph vessel d ssected free from fas iae and adjoining tissue for a length of about 2 mm In that part the lymph essel was cut halfway through and plit open in th proximal d rection A glass cannula with an 0 3—0 4 mm outer diameter and w th a blunt end was mounted on a sho t polyethylene cathet r with about 0 mm of the glas protruding The glass part was s liconiz d with S licone Repelcote (Hopk n and Williams Ltd England) The cannula was introduced into the lymph vessel in d stal d ection for about 10 mm and faste ed to the skin by spraying the wound with Nobecutan® (Bofors Nobel Pharma Sweden)

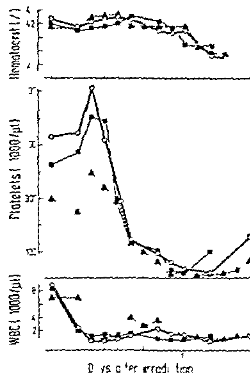


Fig 1 Development of hematocrit of blood platelet level and of white blood cell count (WBC) in 3 rabbits before and during the first 15 days after whole body irradiation with 660 rads (Animal indicated by ■—■ is the same as the one in Fig 4 a and animal indicated by O—O the same as the one in Fig 3 b)

The polyethylene catheter was then cut near the glass cannula and the drops of lymph produced at its end collected into heparinized capillary tubes A slight pressure with a gauze pad was applied now and then on the ear distally to the wound in order to accelerate the formation of a drop of lymph at the cut end of the cannula thereby preventing clotting of lymph in the cannula About 1/2 h after finishing the cannulation a lymph sample was transferred to a hemocytometer and duplicate determination of the red cell concentration carried out under a phase contrast microscope Two more samples of lymph were collected about 1/2 h after collecting the first one When the red cell content of these second samples differed considerably from that of the first set further samples were collected and examined until a reasonably constant level of red cells had been established The whole procedure usually lasted for about 2 h

One ear was used for injections of anesthesia and for blood sampling Reliable lymph sampling could then usually be carried out in the other ear only At first only one successful cannulation of the appropriate ear lymph vessel was achieved in each animal With experience however it became possible to remove the lymph vessel cannula containing coagulated lymph after 24 h, and to insert a new one in the same vessel opening The tip of the cannula could then easily be put beyond the skin area around the wound where signs of slight inflammation were sometimes visible Alternatively an opening was made distally on the same lymph vessel which was now slightly dilated The cannulated ears were otherwise normal in appearance throughout the experiments In this way successful cannulations of the same lymph vessel could be performed on as much as 6 consecutive days

The lymph flow from the cannulated vessel which varied from one cannulation to another and also from one animal to another ranged from almost nothing to about 10 µl/min Total lymph flow per time interval through the cannula was not systematically measured since lymph collaterals were not ligated

Results

Ear lymph from normal animals Peripheral lymph collected from the ears of normal rabbits showed a red cell content ranging from 1000/µl down to almost zero This is in accordance with findings in peripheral lymph from sheep (Smith *et al* 1970)

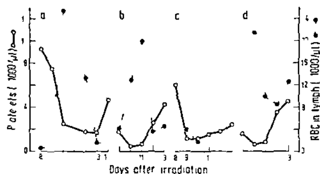


Fig 2 Levels of red blood cells in ear lymph ● ● and of circulating platelets in blood O—O from 4 rabbits some days after whole body irradiation Definite leakage of red blood cells to lymph present in all 4 animals

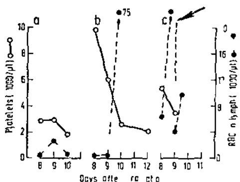


Fig 3 Levels of red blood cells in ear lymph ● ● and of circulating platelets in blood O—O in 3 rabbits some days after whole body irradiation Definite leakage of red blood cells to lymph present in all 3 animals (In rabbit C a platelet transfusion was given on day 9 as indicated by arrow)

Repeated cannulations with lymph collection on five consecutive days in one normal rabbit gave values for lymph red cells ranging from 600 to 1025/μl

Development in irradiated animals The level of circulating blood platelets showed an initial rise after irradiation but from the 5th day and onwards a progressive thrombocytopenia developed The lowest values for circulating blood platelets were reached on about the 10th day after irradiation

In all of the 11 irradiated rabbits the level of circulating blood platelets fell below 35 000/μl and in most cases below 20 000/μl The blood platelet level remained at such a low level for 2 or more days before the number of platelets again started to rise The development of the blood platelet level the white cell count and the hematocrit value in three individual irradiated rabbits are given in Fig 1

In the ear lymph from 7 of the 11 animals (Fig 2 and 3) an increased number of red cells was temporarily observed This increase did always occur during the period with most marked thrombocytopenia and was followed by a gradual normalization as the thrombocytopenia receded In some of the 7 animals this gradual normalization of the erythrocyte admixture to the lymph began one or two days before the number of circulating blood platelets started to rise

The level of red cells in ear lymph from the 4 remaining animal did never ex

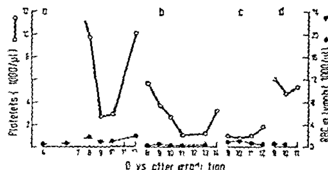


Fig. 4. Levels of red blood cells in ear lymph ●—● and of circulating platelets in blood O—O from 4 rabbits some days after whole body irradiation. None of these animals showed any definite leakage of red blood cells to lymph.

ceed 1000/µl (Fig. 4) and was thus not definitely above the upper normal value. What could be the reason for the observed partition of animals into two groups: a major one with an increased red cell content in ear lymph during thrombocytopenia and a smaller one without such an increase? The animals in the former group did neither develop a more marked thrombocytopenia (Table I) nor a more marked leukopenia (Table II) than did those in the other group.

There was thus no obvious reason why 7 thrombocytopenic animals did develop a demonstrable erythrocyte leakage to their ear lymph, whereas the remaining 4 did not. However, when carefully following the weight development of the animals (Fig. 5), one could again observe that they fell into the same two groups. All the

Table I. Degree and time of occurrence of maximal thrombocytopenia in 11 rabbits exposed to whole body irradiation. Animals divided into 2 groups: those with and those without an increase number of erythrocytes in their ear lymph

	Red cell level in lymph increased	Red cell level in lymph not increased
Number of animals	7	4
Minimum value of platelet in blood × 1000/µl (Mean value and range)	16 (5–33)	21 (6–36)
Days after irradiation when platelet number was at minimum (Mean value and range)	10.4 (9–13)	10.0 (9–11)

Table II. Mean levels of white blood cells (WBC) in blood from 10 rabbits on days 9–11 after whole body irradiation. Animals divided into 2 groups according to lymph content of red blood cells

	Red cell level in lymph increased	Red cell level in lymph not increased
Number of animals	6	4
WBC. Mean and range of counts × 100/µl	12 (3–34)	9 (3–31)

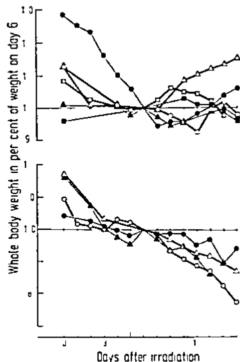


Fig 5 Changes in body weight in 10 rabbits after whole body irradiation (660 rads with ears shielded) All animals had marked thrombocytopenia 9—13 days after irradiation.
Above Weight development in 6 rabbits where there occurred an increased level of erythrocytes in the ear lymph
Below Weight development in another 4 rabbits in which no definite increase in the lymph level of erythrocytes occurred
With the weight levels on day 6 as reference values the weights in the two groups were significantly different from day 9 and onwards ($p = 0.01$ Wilcoxons two sample test) The weight on day 6 was chosen as the "100 %" level because a definite reduction in the number of circulating blood platelets was first observed on that day (see Fig 1)

animal did loose weight in the first period after the irradiation In the animal in which an increased level of erythrocytes was observed in the lymph this weight loss was interrupted by a period of weight gain This period started when the number of blood platelets was low In the second group where the lymph remained normal as regards the level of erythrocytes one could not observe any such temporary weight increase

The lymph flow appeared to be more abundant in animals with the described weight increase and an increase in interstitial fluid volume is therefore a probable explanation for the weight gain It thus appears that a moderate degree of oedema and an increased number of erythrocytes in lymph are correlated phenomena

One of the thrombocytopenic animals with a markedly increased red cell content in the lymph was given a transfusion with a platelet suspension consisting of 10^{10} platelets in 2 ml of saline The platelets had been prepared and treated as described by Abrahamsen (1965) The lymph was normalized as far as red cell content is concerned in about 25 min after the beginning of the transfusion (Fig 3 c)

Discussion

When studying vascular erythrocyte leakage during thrombocytopenia the observation of ear lymph offers some advantages over the observation of thoracic d

lymph. Firstly ear lymph is drained from a tissue which can be shielded during irradiation if this is the procedure used to induce thrombocytopenia Since there is no bone marrow in the ears such shielding will not interfere with the thrombocytopenic effect of irradiation

The experiments reported show that during irradiation induced thrombocytopenia an increased number of red cells may gain access to peripheral lymph distal to the first lymph node During thrombocytopenia the erythrocytes do probably gain access to the interstitium by socalled diapedesis (Van Horn and Johnsson 1966) In the present experiments there were usually no visible petechiae in the rabbit's ears not even when the lymph contained an increased number of erythrocytes It is probably therefore that the red cells in the lymph have arrived there as the result of relatively few erythrocytes penetrating blood vessel walls at each site of leakage

The number of red cells in the ear lymph of the irradiated rabbits of the present investigation did never reach such high levels as has been reported for thoracic duct lymph in thrombocytopenic animals (Woods *et al* 1953) It is probably significant in this connection that the ear lymph in the present experiments was drained only from tissues with capillaries having a socalled continuous type of endothelium The thoracic duct will contain lymph also from tissues with more permeable capillaries such as those of liver and intestinal mucosa

Besides being lymph from mainly one type of tissue the ear lymph is collected much closer to where it is produced than is thoracic duct lymph The time lag from the actual erythrocyte diapedesis to the point where the red cells can be observed in collected lymph should on the average be considerably shorter in ear lymph than in thoracic duct lymph One of the present observations is relevant in this connection When one of the animals with thrombocytopenia and an increased number of red blood cells in the lymph was transfused with a platelet suspension the red cell content of ear lymph was normalized in about 25 min (Fig 3 c Others have found that it takes about 5—6 h after a platelet transfusion before the thoracic duct lymph of thrombocytopenic dogs becomes clear Woods *et al* 1953)

Two aspects of the present results need further consideration Firstly an increased number of red cells in lymph could be observed only in 7 out of 11 animals with similarly induced and similarly developing thrombocytopenias Secondly this erythrocyte appearance in lymph was not the only sign of capillary defect in these 7 animals They also showed an intermittent weight increase during their thrombocytopenic period, a phenomenon which was again lacking in the 4 remaining animals It appears very probable that this intermittent weight increase is reflecting a moderate degree of oedema Some recent observations do furthermore indicate that capillary permeability to protein is increased in such thrombocytopenic animals at the time when they show both the raised level of red blood cells in their peripheral lymph and the moderate and intermittent weight increase Aursnes 1975 It appears therefore that a generalized vascular defect with erythrocyte leakage a somewhat increased capillary permeability to protein and a moderate oedema may develop during marked thrombocytopenia.

Why then could neither the erythrocyte leakage to lymph nor the moderate oedema be demonstrated in 4 of the severely thrombocytopenic animals? It appears that there must have been some unknown individual difference between the animals in the two groups those with and those without a definitely increased number of red cells in their lymph Vascular wall affection of a certain degree might be necessary for the defect to present itself in the form of definite erythrocyte leakage to the lymph This degree might have been reached in the 7 animals and not in the 4 In 2 of the 4 rabbits there was actually some increase in the lymph erythrocyte level during maximal thrombocytopenia but this level never exceeded the upper level observed in non irradiated rabbits (Fig 4)

Since the ears were shielded during exposure the vascular defect developing in this region, and reflected in erythrocyte leakage to the lymph appears to be due to changes in the blood If thrombocytopenia is the only relevant change in this respect then there are various possible explanations for this defect not presenting itself in 4 of the animals *The most likely explanation is that the bone marrow starts to* regenerate blood platelets on different days after irradiation in the various animals The first few new platelets produced may not influence the level of circulating blood platelets very much but may be sufficient for some endothelial recovery New platelets are known to be hemostatically more efficient than old ones (Karpatkin 1972) and the former are therefore probably also more efficient in preventing red cell leakage to the lymph The endothelium could then have started to recover before a stage with erythrocyte leakage had been reached and also before there was any measurable increase in the level of circulating platelets Such an explanation is supported by the following observation In animals with increased numbers of red cells in the lymph this lymph abnormality was sometimes normalized one or two days before the level of circulating blood platelets started to rise

It appears from the present experiments that the induction of a vascular wall defect during thrombocytopenia is not only a question of the level of circulating blood platelets being reduced below a certain limit Moreover the vascular wall defect developing during thrombocytopenia manifests itself not only as an increased extravasation of red cells *More general permeability changes* leading to some slight oedema do apparently also take place

The radiation exposures have been performed at the Norwegian Radium Hospital Department of Medical Physics I am ry grateful for this help The present work has been support d by grants from Hjelpest kk nes Medisinske Forskningsfond The Nansen Foundation and The Norwegian Research Council fo Sc ence and the Humanities All this support is gratefully acknowledged

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From the Department of Pharmacology, Odense University, Denmark

Potentiation of Anaphylactic Histamine Release from Guinea Pig Lung by Maleate and Succinate

By

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Abstract

CHAKRAVARTY, N. and H. J. SORENSEN. *Potentiation of anaphylactic histamine release from guinea pig lung by maleate and succinate.* Acta physiol. scand. 1973. *88*. 401—411.

Both maleic acid and succinic acid enhance anaphylactic histamine release from guinea pig lung *in vitro*—the degree of enhancement being of the same order of magnitude. The stimulating effect of both the substrates is inhibited by relatively low concentrations of malonic acid and bromopyruvic acid, and no stimulation occurs in response to either substrate when histamine is released by the antigen in an anaerobic medium. Maleic acid is shown to be metabolized to CO_2 by guinea pig lung nearly at the same rate as the tissue oxidizes succinic acid to CO_2—the conversion of both the substrates to CO_2 being inhibited by malonic acid. Furthermore, D(+)malic acid has been identified as an intermediate product in the metabolism of maleic acid by guinea pig lung. It is suggested that maleic acid follows the same metabolic pathway in guinea pig lung as revealed earlier in rabbit kidney *viz.* maleic acid → D(+)malic acid → oxaloacetic acid → CO_2. The findings thus suggest that both maleic acid and succinic acid enhance anaphylactic histamine release from guinea pig lung through their metabolic utilization in the tricarboxylic acid cycle.

The enhancement of anaphylactic histamine release from guinea pig tissue by succinate was first reported by Moussatche and Prouvost Danon (1957, 1958) and has since been confirmed in several laboratories (Yamasaki *et al.* 1960, Austen and Brocklehurst 1961, Chakravarty 1962, Diamant and Fredholm 1963). The enhancement has been shown to occur only in presence of an adequate supply of oxygen and to be inhibited by a relatively low concentration of malonate, suggesting that oxidative metabolism of succinate is linked with the process (Chakravarty 1962). However, maleic acid (cis butenedioic acid, a stereoisomer of fumaric acid) which was generally considered to be metabolically inert, has also been shown to stimulate anaphylactic histamine release from guinea pig tissue by Austen and Brocklehurst (1961). On the basis of this observation the latter authors thought that the potentiation of anaphylactic histamine release by succinate could not be attributed to its metabolism in the tricarboxylic acid cycle. Since certain mammalian tissues can metabolize maleic acid (Taggart, Angielski and Morell 1962), it was felt that the

mechanism of the potentiating action of succinate and maleate on anaphylactic histamine release fom guinea pig tissue should be re-evaluated The experiments reported here suggest that the potentiation of anaphylactic histamine release caused by both succinate and maleate is related to their oxidative metabolism in the tri carboxylic acid cycle

Material and Methods

Male guinea pigs 250—350 g were sensitized to egg albumin as described previousl (Chakravarty 1960 and used for the experiments 4—16 weeks after the first dose of egg albumin. Lungs from 2 guinea pigs were used for each experiment. The animals were stunned by a blow on the head and bled from the axillary artery The heart and the lungs were then removed from the thoracic cavity and the lungs were perfused with Krebs-Ringer solution through the pulmonary artery to wash away blood The lungs were divided into 10—13 equal parts—pooling each sample (350—400 mg from both the guinea pigs The lung pieces were quickly dried with a filter paper and weighed in a torsion balance The individual samples were then cut into fragments 1—2 mm × 1—2 mm × 0 75 mm (thickness) weighing about 1 mg with McIlwain tissue chopper The samples were washed and incubated with 5 ml Krebs-Ringer solution 139 8 mM NaCl 4 7 mM KCl 1 2 mM $MgSO_4$ 2 5 mM $CaCl_2$ 2.5 mM Na_2PO_4 0 6 mM KH_2PO_4 final pH 7 2 at 37 C under mild shaking The antigen was added after prewarming for 6—10 min When substrates and/or inhibitors were used the tissue was incubated with them (sodium salts for the same length of time (6—10 min) before exposure to antigen After incubation with antigen for 10—12 min the incubation fluid was separated from the lung fragments The remaining histamine in the tissue was then released by boiling the lung fragments in 0 9 NaCl for 3 min Histamine released by antigen antibody reaction into the incubation fluid and the remaining histamine were then determined by the biological method using guinea pig ileum in an organ bath Code and McIntire 1956) or the fluorometric method Shore *et al* 1959 The percentage of the total histamine content of the samples released by anaphylactic reaction *in vitro* has been presented in the tables and figures There was a variation in the lung histamine base content of the guinea pigs—ranging from 43 to 20 4 g g lung in different experiments The spontaneous histamine release in the absence of antigen was usually 0 4—0 6 but in the anaerobic experiments the spontaneous release was 2— 5 The spontaneous release has not bee deducted from the values presented

The metabolic experiments in which the production of CO_2 by guinea pig lung *in vitro* from labeled substrates was determined were performed by a modification of the technique used by Cupp and Crevasse 1963 The lung tissue was prepared as described above and incubated in 2 ml Krebs-Ringer solution with the substrates at 37 C under shaking for 30 min ^{14}C labeled maleic acid and succinic acid were used as substrates ^{14}C maleic acid was purified by two dimensional thin-layer chromatography on silica gel plates using I diisopropyl ether formic acid water—90 / 3 and II benzene methyl alcohol acetic acid—90 16 8 as the solvents ^{14}C maleic acid was identified after chromatography by autoradiography eluted with water and used after freeze-drying A small glass tube containing hyamine hydroxide which trapped $^{14}CO_2$ was transferred to a scintillation flask containing 0 4 omnifluor in toluene and counted in a *Beckman scintillation spectrometer* Quenching was corrected by internal standardization Controls run with ^{14}C labeled maleic acid and succinic acid without tissue were always included and gave some count 103— 00 in hyamine hydroxide These values were only 1 15 to 1 30 of the values for $^{14}CO_2$ production from substrates incubated with the tissue and have been subtracted to obtain the true CO_2 production by the tissue

In the experiments in which the oxidation of ^{14}C maleate to $^{14}CO_2$ by rat mast cells was studied the same method with a smaller ionized flask was used Pure populations of mast cells from Sprague Dawley rats (400—450 g were isolated for these experiments in concentrated serum albumin as described previousl (Chakravarty and Zeuthen 1965) The purity of the isolated mast cells was 99

Experiments were also performed in an attempt to determine and identify intermediate product(s) in the oxidative metabolism of maleic acid ^{14}C maleic acid was purified by two-dimensional thin-layer chromatography as described above Guinea pig lung fragments were then incubated with the purified substrate at 37 C for 1 h The reaction was interrupted by cooling to 0—4 C The incubation fluid was separated from the lung fragments and applied to an anion exchange column 15 cm × 1 cm Dowex 1 X8 50 100 mesh in formate form The column was then washed with 100 ml water and eluted with 250 ml 8 N formic acid The

TABLE I Potentiation of anaphylactic histamine release by maleate and succinate

Expt no	Substrate	Conc mM	Histamine release
1	0	—	24 0 ± 0 65 (4)
	Maleate	1	37 2 ± 0 37 (4)
2	0		11 0 ± 0 37 (3)
	Maleate	1	25 0 ± 0 87 (3)
3	0		21 4 ± 0 26 (4)
	Maleate	5	30 2 ± 1 83 (3)
4	0		28 8 ± 0 99 (3)
	Maleate	5	45 1 ± 1 98 (4)
5	0		19 9 ± 0 47 (3)
	Maleate	5	31 5 ± 0 47 (4)
6	0		21 8 ± 0 95 (4)
	Succinate	1	33 1 ± 1 06 (4)
7	0		15 1 ± 0 37 (4)
	Succinate	5	24 6 ± 1 47 (4)

Mean values ± S E Number of samples in parentheses
$P < 0\ 005$ for all the expts

eluate was freeze-dried thus with simultaneous removal of formic acid The dried residue was dissolved in 100—400 μl 80 ethyl alcohol and chromatographed on silica gel plates with a mixture of ethylacetate 210 ml and formic acid 30 ml the mixture being saturated with water Radioactivity in the dried plate was identified by autoradiography Known standards of Krebs cycle intermediates—both radioactive and non radioactive—were run parallel with the sample The non radioactive organic acids were identified by spraying with a 0 04 % solution of bromo cresolblue in 96 ethyl alcohol

1 4 ^{14}C and 2 3 ^{14}C maleic anhydride 1 4 ^{14}C and 2 3 ^{14}C succinic acid and L-malic acid-C^{14} (U) were obtained from The Radiochemical Centre Amersham Maleic anhydride was converted to maleic acid before use Omnifluor a precisely blended mixture of 98 % PPO and 2 p-bis (O methylstyryl) benzene was purchased from New England Nuclear Chemicals Thin layer chromatography plates precoated with 0 25 mm layer of silica gel were purchased from E Merck AG Maleic acid and succinic acid were obtained from E Merk AG Maleic acid was found by thin layer chromatography on silica gel plates using different solvents to be free from any detectable amount of succinic acid Malonic acid D(+)malic acid DL-malic acid and bromopyruvic acid were obtained from Sigma Chemical Co

Results

Potentiation of anaphylactic histamine release by maleate and succinate

Table I shows the potentiation of anaphylactic histamine release from guinea pig lung *in vitro* by maleate and succinate in 1—5 mM concentration The potentiation by maleate varied from 34—126 % the highest value being in the experiment in which the anaphylactic histamine release in a substrate free medium was the lowest (Expt 2) The average potentiation of histamine release by maleate was 63 % which is of the same order of magnitude as for the average succinate induced potentiation *i e* 58 % In order to compare the stimulating effect of succinate and maleate on histamine release the two substrates were also used in the same experiment as shown in Fig 1 maleate and succinate produced about the same degree of potentiation

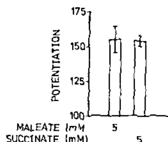


Fig 1 The Figure shows that maleate and succinate used in the same experiment are about equally effective in producing potentiation of anaphylactic histamine release Potentiation 100 on the ordinate represent histamine release in the absence of a substrate (18 8 % of the total histamine content) and the release with maleate and succinate is expressed as a percentage of the rel ase in their absence Four samples each for maleate and succinate P > 0 5

Inhibition of maleate induced potentiation of anaphylactic histamine release

Table II shows the effect of malonate on maleate potentiation of anaphylactic histamine release 10 mM malonate did not inhibit histamine release in the absence of a substrate but was consistently effective in inhibiting the potentiation caused by 1–5 mM maleate (Expt 1—3) 20 mM malonate on the other hand caused an inhibition of histamine release both in the absence and the presence of maleate

Bromopyruvate is an irreversible inhibitor of succinate dehydrogenase (Sanborn *et al* 1971) The effect of this inhibitor on maleate potentiation of histamine release is shown in Table III While 1 mM bromopyruvate had a direct inhibitory effect on histamine release in the absence of a substrate 0 1 mM concentration of the inhibitor was without such effect The latter concentration however produced a significant inhibition of maleate induced potentiation of histamine release (Expt 4) Expt 5 in the same table points to the expected inhibitory effect of 0 1 mM bromopyruvate on succinate induced potentiation of histamine release

TABLE II Inhibition of maleate induced potentiation of anaphylactic histamine rel ase by malonate

Expt no	Maleate mM	Malonate mM	Histamine release	
1	0	0	11 0±0 37 (3)	p > 0 4
	0	10	10 4±0 52 (3)	
	1	0	25 0±0 87 (3)	p ~ 0 05
	1	10	17 0±2 32 (3)	
2	0	0	11 9±0 73 (4)	
	5	0	24 5±0 27 (4)	p < 0 001
	5	10	18 2±0 55 (4)	
3	0	0	24 1±0 77 (4)	
	5	0	40 8±4 67 (4)	p < 0 025
	5	10	24 2±2 20 (4)	
4	0	0	25 7 (2)	
	0	20	8 5±1 38 (3)	
	5	0	33 9 (2)	
	5	20	13 6±2 91 (3)	

Mean values ±S E Number of samples in parentheses

TABLE III Inhibition of maleate and succinate potentiation of anaphylactic histamine release by bromopyruvate

Expt no	Substrate	Bromopyruvate mM	Histamine release	
1	0	0	16 6±1 1 (4) }	p < 0 001
	0	1	9 0±0 3 (4) }	
2	0	0	20 9 (2)	
	0	0 1	20 2 (2)	
3	0	0	23 8 (2)	
	0	0 1	21 9 (2)	
4	0	0	19 9±0 5 (3)	
	Maleate 5 mM	0	31 5 ±0 5 (4) }	p < 0 001
	Maleate 5 mM	0 1	24 8 ±1 2 (4) }	
5	0	0	23 8 (2)	
	Succinate 5 mM	0	32 9 (2)	
	Succinate 5 mM	0 1	28 1 (2)	

Mean values ±S E Number of samples in parentheses

TABLE IV Effect of maleate and succinate on anaerobic glucose dependent anaphylactic histamine release

Expt no	Substrate	Histamine release	
1	Glucose 5 mM	10 9±0 3 }	P > 0 05
	Glucose 5 mM+succinate 5 mM	8 6±1 0 }	
2	Glucose 5 mM	11 3±0 6 }	P > 0 5
	Glucose 5 mM+maleate 5 mM	11 5±1 2 }	

Mean values ±S E of 3 samples Anaphylactic histamine release was completely blocked in the absence of glucose in the anaerobic medium

TABLE V Incubation of C maleate metabolite with minced guinea pig lung

Additional substrate	C-Metabolite → CO (CPM)[b]	
	Expt 1	Expt 2
0	861	1739
L(−) Malic acid 2 mM	545	912
D(+) Malic acid 2 mM		369
DL— Malic acid 4 mM	325	

[a] 1 h at 37 C with 1 g lung
[b] Corrected for background and quenching The amount of metabolite added corresponded to 57 000 CPM in Expt 1 and 105 500 CPM in Expt 2

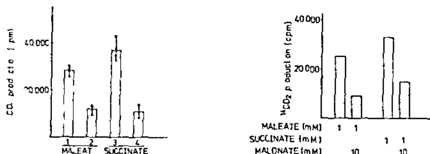


Fig 2 Production of $^{14}CO_2$ from 1 4 ^{14}C maleate (no 1) 2 3 ^{14}C maleate (no 2) 1 4 ^{14}C-succinate (no 3) and 2 3 ^{14}C succinate (no 4) incubated with guinea pig lung fragments *in vitro* for 30 min Concentration of maleate and succinate 5 mM specific activity 0 045—0 088 μCi/μmol CPM values corrected for background and quenching are expressed per g tissue per μCi radioactive substrate in the incubation fluid Values from 3—4 expts are presented

Fig 3 Inhibition of $^{14}CO_2$ prodution from 1 4 ^{14}C maleate and 1 4 ^{14}C succinate by malonate Guinea pig lung fragments were incubated *in vitro* for 30 min with the substrates (Conc 1 mM specific activity 0 35—0 48 μCi/μmol) and the inhibitor as shown in the figure CPM values corrected for background and quenching are expressed per g tissue per μCi radioactive substrate in the medium

Effect of maleate and succinate on anaerobic glucose dependent histamine release

The anaerobic experiments were performed with lung fragments suspended in physiological salt solution essentially of the same composition as described under methods and containing 5 mM glucose Instead of phosphate buffer however 10 % (v/v) tris buffer was added giving a final pH 7 6 Anaerobiosis was produced in Warburg apparatus by pre incubating for 30 min at 37° C under nitrogen (> 99 95 %) before exposure to the antigen As shown in Table IV histamine release in the anaerobic medium was not stimulated by succinate or maleate

Metabolism of maleate by guinea pig lung

As shown in Fig 2 maleate is readily oxidized by guinea pig lung to CO_2 the rate of oxidation being similar to that for succinate The metabolism of maleate to CO_2 is inhibited by 10 mM malonate (Fig 3) Here again the malonate inhibition of the

TABLE VI Metabolism of maleic acid by rat mast cells

Expt no	Maleic acid mM	Specific activity mCi/mM	1 4 ^{14}C-Maleic acid → $^{14}CO_2$ CPM/10⁶ cells
1	0 55	2 2	175
2	0 59	3 5	346

Incubation time with mast cells 30 *min*
CPM from $^{14}CO_2$ in Expts 1 and 2 expressed per 10^6 cells per μCi ^{14}C-maleic acid in the incubation fluid are 79 and 83 respectively CPM values are corrected for background and quenching

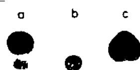


Fig 4 Autoradiograph of thin layer chromatogram showing a metabolite of ^{14}C maleate produced by incubation with guinea pig lung fragments *in vitro* Maleate conc 0 036 mM specific activity 11 μCi/μmol a Incubation fluid after ion exchange chromatography b ^{14}C malic acid c Control ^{14}C-maleic acid incubated without lung tissue The metabolite (lower spot in a) corresponds to malic acid

oxidation of maleate to CO appears to be of the same order of magnitude as the inhibition of succinate oxidation to CO

When guinea pig lung was incubated with ^{14}C maleic acid we could identify a metabolic product in the incubation fluid as malic acid One of three such experiments is shown in Fig 4 The incubation fluid (a) showed two spots The R value of the lower spot coincided with that of malic acid (b) The upper spot corresponded to maleic acid (c) which was run as a parallel control without tissue In 2 other expts in which ^{14}C maleic acid was incubated with lung tissue in presence of 20 and 40 mM malonate the metabolite (malic acid) did not appear However in another experiment bromopyruvate (2 mM) did not prevent the appearance of the metabolite

In order to determine if the intermediate product of maleic acid isolated from the incubation fluid and identified by chromatography to be malic acid was dextro or laevo form the experiments shown in Table V were done The metabolite from the thin layer chromatography plate was eluted with 0 9 % sodium chloride solution and incubated with minced guinea pig lung The ^{14}C metabolite isolated from the incubation fluid of two experiments was incubated as shown in the table with either no addition substrate or with non radioactive L(–) D(+) or DL malic acid The incubation was carried out at 37° C for 1 h and the radioactivity in CO produced was determined When the metabolite would correspond to the added non radioactive substrate one would expect a reduction in the counts for CO because of the diluting effect of the non radioactive substrate It may be seen in the table (Expt 2) that the most marked reduction in the counts in CO—to 20 % of the original level—occurred when the metabolite was incubated with D(+) malic acid There was also a pronounced reduction in the counts with DL malic acid (Expt 1) These observations point to the metabolite as D(+) malic acid L(–) malic acid caused some reduction in the counts of CO This could be because L(–) malic acid was slightly contaminated (8 %) with D(+) malic acid It is also possible that L(–) malic acid may have some inhibitory effect on the oxidation of D(+) malic acid in the same way as L(–) lactate is a weak competitive inhibitor of D(+) lactate oxidation (Tubbs and Greville 1961)

Since only a small fraction of guinea pig tissue is represented by mast cells it is difficult to say if the metabolism of maleic acid occurs in the mast cells or other cells Unfortunately a free suspension of pure guinea pig mast cells cannot be obtained for this study We have therefore used rat peritoneal mast cells instead for this purpose $4\ 7 \times 10^6$ to $5\ 1 \times 10^5$ mast cells were incubated with 0 55—0 59 mM maleic acid

(specific activity 2.2—3.5 mCi/mM) for 30 min at 37° C. The quantity of $^{14}CO_2$ produced expressed as CPM is shown in Table VI. Owing to a difference in the sites of labeling of the carbon atoms, the oxidation ($^{14}CO_2$ production) of maleic acid and of other substrates could not be directly compared. Our observations show, however, that the rate of metabolism of 1,4-^{14}C maleic to $^{14}CO_2$ by rat mast cells is *of the same order of magnitude as the oxidation of uniformly labeled* ^{14}C glucose to $^{14}CO_2$ by these cells. The average rate of conversion of 1,4-^{14}C maleic acid to $^{14}CO_2$ by rat mast cells was 77.5 CPM per 10^6 cells per μCi ^{14}C in the incubation fluid (maleic acid conc. 0.55—0.59 mM). As 10^6 mast cells correspond to about 1 mg wet weight assuming the average specific gravity to be 1 (Chakravarty and Zeuthen 1965), the above-mentioned rate of $^{14}CO_2$ production corresponds to the oxidation of maleic acid by about 1 mg mast cells. This gives a basis for comparison with the metabolism of maleic acid by guinea pig lung. With 1 mM maleic acid conc. in the incubation fluid, the rate of conversion of 1,4-^{14}C maleic acid to $^{14}CO_2$ by guinea pig lung fragments was 25,200 CPM/g lung per μCi ^{14}C in the medium (see Fig. 3). *Expressed per mg wet weight this amounts to* 25.2 CPM for guinea pig lung. Thus the production of $^{14}CO_2$ from ^{14}C maleic acid by rat mast cells mentioned above (77.5 CPM/mg) was 3 times higher than that by guinea pig lung, although the maleic acid concentration in the medium was about 75% higher for guinea pig lung. Thus it is possible that guinea pig mast cells may metabolize maleic acid to CO_2, although other types of cells in guinea pig lung may also have this property.

Discussion

Our results show that maleate in 1—5 mM concentration causes a remarkable enhancement of anaphylactic histamine release from guinea pig lung *in vitro*, the degree of enhancement being of the same order of magnitude as for succinate. Succinate is the only Krebs cycle substrate known to have a unique stimulating effect on anaphylactic histamine release from guinea pig lung. Austen and Brocklehurst (1961) reported a pronounced enhancement of anaphylactic histamine release from guinea pig lung by 5 mM α-ketoglutarate but 0.5 mM had very little effect. Chakravarty (1962), however, found 10 mM α-ketoglutarate ineffective in stimulating histamine release, and Yamasaki *et al.* (1960) reported only a mild enhancement with this substrate (3—40 mM). The stimulation caused by succinate is seemingly related to its oxidative metabolism in the tricarboxylic acid cycle, as shown by the dependence of the stimulation on oxygen and its inhibition by a relatively low concentration of malonate (Chakravarty 1962). The present findings show that maleate-induced potentiation of anaphylactic histamine release occurs only in presence of oxygen, the anaerobic glucose-dependent histamine release being uninfluenced both by maleate and succinate. In addition, maleate-induced potentiation is inhibited by relatively low concentrations of malonate and bromopyruvate, both inhibitors of succinic dehydrogenase. Malonate in higher concentrations (20—60 mM) has an

inhibitory effect on anaphylactic histamine release from guinea pig lung even in the absence of a substrate (Moussatche and Prouvost Danon 1958) But 10 mM malonate which has no effect on histamine release in a substrate free medium has been shown to inhibit the potentiation caused by maleate (see Table II) the result being very similar to that reported earlier for malonate inhibition of succinate induced stimulation of histamine release (Chakravarty 1962)

Maleate has lately been shown to be capable of being metabolized to carbon dioxide by rabbit kidney (Taggart *et al* 1962) This raises the possibility that maleate may also be metabolized by other mammalian tissues Guinea pig lung has indeed been shown in the present investigation to oxidize maleate to CO_2 *in vitro* at about the same rate as the tissue oxidizes succinate to carbon dioxide In an attempt to explore the pathway of the conversion of maleate to carbon dioxide the incubation fluid in which guinea pig lung fragments were incubated with ^{14}C maleate was analyzed Autoradiography of the thin layer chromatogram revealed a metabolite of maleate having the same R_f value as for malic acid When this metabolite was eluted and incubated with minced guinea pig lung in presence of non radioactive malic acid the result indicated that the metabolite was D(+) malic acid Guinea pig lung thus seems to be another source of maleate hydratase which converts maleic acid to D(+) malic acid This enzyme has already been described in the rabbit kidney (Englard *et al* 1967) D(+) malic acid can also be oxidized to CO_2 in mammalian tissue It can enter the tricarboxylic acid cycle through the activity of D 2 hydroxy acid dehydrogenase (Tubbs and Greville 1961 Camma 1969) or succinic dehydrogenase (Dervartanian and Veeger 1965) either of which converts it to oxaloacetate

Our observations on the metabolism of maleate do not permit us to come to a conclusion about the pathway for its complete oxidation in guinea pig lung But the observations that guinea pig lung can convert maleate to carbon dioxide at a rather fast rate and that D(+) malate is an intermediate product would be consistent with the pathway described before for rabbit kidney viz maleic acid → D(+) malic acid → oxaloacetic acid → CO_2 We have shown that malonate (10 mM) and bromopyruvate (0.1 mM)—both inhibitors of succinic dehydrogenase—inhibit the maleate induced enhancement of anaphylactic histamine release Furthermore the oxidation of maleate to carbon dioxide is inhibited by malonate (10 mM) practically to the same extent as succinate oxidation is inhibited by it It is thus tempting to suggest that succinic dehydrogenase may be primarily active in guinea pig lung for the metabolism of D(+) malate We cannot rule out however D 2 hydroxy acid dehydrogenase because the sensitivity of this enzyme to malonate is unknown to us Malonate (20–40 mM) also blocked the earlier step of conversion of maleate to malate presumably through an action on maleate hydratase Considering the structural similarity of malonic acid and maleic acid it is conceivable that malonate may exert this action through the inhibition of maleate hydratase

D(+) malate added a few minutes before the exposure of minced guinea pig lung to antigen did not stimulate anaphylactic histamine release in our experim

Moussatche and Prouvost Danon (see Moussatche 1960) and Yamasaki *et al* (1960) however reported a stimulation of histamine release with malic acid after incubation for a longer period but no information is given if the substrate used was L(−) DL or D(+) form The lack of stimulation after a brief exposure to D(+) malate in our experiments may be due to lack of penetration of the substrate Lack of oxidation of exogenously applied Krebs cycle substrates by leucocytes due to lack of penetration has been previously reported (Martin *et al* 1955) We have no knowledge about the penetration of D(+) malic acid into the cells concerned in histamine release viz the mast cells Nevertheless maleic acid is oxidized to CO both by guinea pig lung and by pure populations of rat peritoneal mast cells Thus it seems likely that maleic acid penetrates the mast cells of both the species and D(+) malic acid produced within the cell may exert a stimulating effect on histamine release

There are evidences supporting the view that anaphylactic histamine release is dependent on oxidative or glycolytic energy metabolism, but how the energy metabolism is coupled to the release process is not known Although our observations indicate that succinate stimulation of anaphylactic histamine release from guinea pig lung is linked with the oxidation of the substrate in the tricarboxylic acid cycle it is not clear which step in the metabolism of succinate may contribute to the stimulation Succinate is the only Krebs cycle substrate which transfers electrons directly to flavoprotein Maleate another enhancer of anaphylactic histamine release is apparently first converted by guinea pig lung to D(+) malate which may be further oxidized to CO through succinic dehydrogenase or D 2 hydroxy acid dehydrogenase It is of interest to note that even the latter enzyme is NAD independent being directly linked to flavoprotein (Cammack 1969)

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The Liver-like Anion Transport System in Rabbit Kidney, Uvea and Choroid Plexus. I. Selectivity of Some Inhibitors, Direction of Transport, Possible Physiological Substrates

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Abstract

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Accumulation of o-iodohippurate and iodipamide *in vitro* occurs by 2 overlapping systems, the liver-like L- and H-, the classical PAH transporting one. Selectivity of competitors is compared by measuring depression of simultaneous uptake of o-iodohippurate and iodipamide. Results (kidney cortex): o-Iodohippurate has similar H-selectivity as hippurate, while iodipamide is much more L-selective than its non-iodinated parent. Ioglycamic acid is at least as L-selective as iodipamide. Rabbit bladder bile is L-selective, rabbit and human urine H-selective. Probenecid is somewhat H-selective, its diethyl homologue more H-selective, its dibutyl homologue more L-selective. Phenol red, furosemide, ethacrynic acid, sulfobromophthalein, phenolphthalein and iopanoic acid are rather unselective. Selectivity of probenecid, hippurate and iodipamide is not influenced by incubation time. Cephaloridin *in vitro* is somewhat H-selective but inhibits both systems in kidney, choroid plexus and uvea. *In vivo* 200 mg/kg causes tubular damage which depresses the H- more than the L-system. Choroid plexus and ciliary processes are undamaged. The two systems L- and H- seem to have the same direction of transport: from aqueous humour and CSF to the blood and from the blood into the urine. The H-system mainly deals with candidates for urinary and the L-system with candidates for biliary excretion.

As recently reported (Barany 1972) there exists a, probably composite, liver-like anion transport system in the kidney cortex, the anterior uvea and the choroid plexus. When these tissues or liver are incubated *in vitro* simultaneously with o-iodohippurate (Hippuran®) and the cholangiographic agent iodipamide (Biligrafin®), a dicarboxylic acid, they accumulate iodipamide despite the presence of (unsubstituted) hippurate in concentrations which completely prevent the simultaneous accumulation of o-iodohippurate. In the following the previously well known organic anion transport system of the three extrahepatic tissues is called the hippurate or H-system while the new one is called the liver-like or L-system (without always repeating that it might be composite). Until now the L-system has been found in one or several tissues of 3 species of monkeys, of the cat, dog, rabbit, guinea pig, rat

and chicken and in the choroid plexus of cattle It has not been looked for in other bovine tissues or in man nor has a systematic search been made all over the body in any species

The 3 substances iodipamide o iodohippurate and hippurate are not really selective for the 2 transport systems neither as test substances nor as inhibitors Iodipamide also has affinity for can saturate and can be accumulated by the H system even if its affinity for that system is roughly 100× less than for the L system The affinity of hippurate for the L system is low However while it is probable that one component of the L system has even less affinity for hippurate than the other some depression of the L-system is caused by excess hippurate and it is also possible (but not proved) that a small fraction of o-iodohippurate uptake is by way of the L system

Probenecid has affinity for both system but less for the liver like one

In the rabbit under our conditions of incubation and with tracer concentrations of the test substances the L system is responsible for about half of the iodipamide uptake in kidney cortex slices for less in anterior uvea and for more in choroid plexu The rest of the uptake is due to the H system

The lack of substances with exclusive affinity for the one or the other of the two systems makes it difficult firmly to establish their physiological and pharmacokinetic importance For this one would like to be able to inhibit the one without affecting the other While it is possible that there are no substances with completely specific affinities there is no reason why the 3 substances selected by guesswork for a start should be the most specific of all A search for more specific inhibitors and test substances is therefore under way

Pending the discovery of more selective substances the present paper reports an attempt by less direct means to get an idea of the direction of transport of the new system and some of its physiological substrates A method for comparing selectivities of inhibitors despite the lack of really selective reference substances is described It is based on the simultaneous use of 2 test substances and applied to some congeners of hippurate and iodipamide to urine and bile to probenecid homologues and to a number of other substances of interest among them cephaloridine Experiments with this antibiotic make it probable that the direction of transport of the L system is the same as that of the H system

Methods

The animal were pigmented or white rabbits of both sexes The methods solutions etc were exactly as described Barany 1972)

The main experiment performed was measurement of simultaneous *in vitro* uptake by kidney cortex slices of the test substances iodipamide and o-iodohippurate (hippuran for shortness) labelled with different iodine isotopes and if not otherwise stated used in very low concentrations Inhibitor in logarithmically spaced concentrations were added to the incubation medium before immersion of the tissue and the degree of depression of uptake of the two test substances measured by comparison with the uptake in 2 control bottles run simultaneously All uptake figures refer to specific uptake the unspecific uptake was corrected for by means a totally inhibited bottle containing 12 6 mM unlabelled iodipamide

Statistics of the method
Data from 34 identically performed unselected consecutive experiments in 34 rabbits all done over a period of about one year by the same technician were used. The rabbits were of both sexes and albino as well as pigmented however 31 expts had 2 independent control bottles 3 had only one The following estimates of the ranges of random variation are based on these control bottles Each of them contained 2 uninhibited kidney cortex slices the specific tissue/medium ratios (T/M) for these 2 slices were averaged and constitute the unit reading

1 *Variation within animals*
The correlation coefficient between T/M for hippuran in bottles 1 and 2 was 0 94 (n = 31) the same value was found for iodipamide The average ratio between bottles two and one was 1 027±0 094 and 1 07±0 14 (S.D., n = 31) for hippuran and iodipamide respectively Thus the ratio between 1 bottle and the average of 2 which is the normal situation for estimation of percent uptake in a single experiment in this paper has a coefficient of variation of 8 ? and 12 % for hippuran and iodipamide respectively The ratio (T/M hippuran)/(T/M iodipamide) also varies between bottles containing slices of the same kidney the coefficient of variation within the animals for one bottle with 2 slices is 8 5

2 *Variation between animals*
The average T/M in bottle one was 7 59±1 75 (S.D n = 34) for hippuran and 14 43±4 15 for iodipamide In bottle two corresponding figures were 7 72±1 93 and 15 33±4.98 (n = 31) The large standard deviations show that slices from different animals vary considerably This matters if after systemic treatment a control animal instead of control slices from the same animal has to be used, as in some experiments with cephaloridine in the present paper With such wide variations the ratio experimental/control tends to be log normally distributed Taking this into account and calculating the variance by pooling variance within groups which used the same batch of test substances and were run within a short time period the standard deviation of the ratio between two uninhibited bottles from different animals was obtained as 0 131 log units for hippuran and 0 164 log units for iodipamide Expressed in the usual percent uptake figures the ±1 S.D range for hippuran and iodipamide therefore are 74—135 and 69—146 % respectively and the ±2 S.D range 55—183 % and 47—213 % Thus a single bottle has to show about 50 % less uptake of a test substance than a single control bottle from another animal for the depression to be significant at the 0 025 level. (For comparisons between single bottles from the same animal the corresponding ±2 S.D range is 81—119 and 72—128

Animals differ not only in absolute uptake but also in the relation between the uptake of the 2 test substances The correlation between T/M for hippuran and iodipamide in the single bottle was only 0 57 (n = 34 P < 0 001) for bottle one and 0 71 (n = 31 P < 0 001) for bottle two Another way of expressing this variation between animals was by forming (T/M hippuran / T/M iodipamide for each bottle The average of this ratio were for bottl one 0 55 n = 34 and for bottle two 0.53 (n = 31 If the statistics are calculated from the logarithms of the ratios instead the means geometric) become 0 55 and 0 52 and the standard deviations 0 073 and 0 082 log units This makes the coefficients of variation for (T/M hippuran / T/M iodipamide computed for different animals using the same batch of test substances 18 % for bottle one and 21 for bottle two or about 20

Theory

1 *The case of perfectly selective inhibitors one imperfectly and one perfectly selective test substance*

In order to be able to identify more selective inhibitors and possibly better test substances among hundreds of compounds it is useful to have a picture of what to look for The two following theoretical sections make simplifying assumptions but try to keep close to actual figures obtained experimentally

At present we have 2 test substances hippuran and iodipamide Let us first assume that we run an uptake experiment with both simultaneously and that in the tissue under study hippuran is a perfectly specific test substance that is transported only by one transport system the H-system while iodipamide is transported by two systems

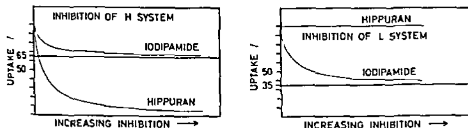


Fig 1 Explanation see text

35 % of maximum uptake in tracer concentrations being due to the H system and 65 % to a different system the L system (The figures are chosen so as to make the graphs clearer but are not very far from the truth for rabbit kidney cortex)

Fig 1 shows how uptake of the 2 test substances would be affected by increasing inhibition of the 2 transport systems separately Inhibition of the L system would not affect hippuran uptake at all and would depress iodipamide uptake at most by 65 % Inhibition of the H system would depress iodipamide uptake by at most 35 % and this only when the H system is completely suppressed

Fig 2 *a* shows a different way of plotting these relations The abscissa represents iodipamide uptake the ordinate hippuran uptake The uninhibited uptake is represented by point A at 100 per cent of both Now gradually increased selective inhibition of the L system would yield points along the heavy horizontal line A—B_1 while gradually increased selective inhibition of the H system would lead to points along A—C_1 The line A—B_1 stops at 35 % and the A—C_1 line at 65 % iodipamide uptake

The above is true for completely selective inhibitors and with one completely selective test substance hippuran It is evident that if hippuran too were a some what unselective test substance *e g* that 10 % of its uptake under uninhibited con ditions were due to the L-system then gradual selective inhibition of the L system would yield a sloping straight line to B instead of B_1 and selective inhibition of the H system would bring hippuran uptake down only to C not C_1

2 *Imperfectly selective inhibitors*

By definition an imperfectly selective inhibitor has affinity to both systems If it is still very specific it can inhibit one system deeply before appreciably inhibiting the other Assume that the specificity is so high that the one system is completely in hibited before inhibition of the other system is noticeable If the inhibitor runs through a sufficiently wide concentration range one would then find a graph A—B_1—B—D for an L inhibitor and a graph A—C—C_1—D for an H inhibitor (Fig 2 *a*) If the inhibitors are less specific the corners at B and C will be rounded The individual selectivity lines inside the parallelogram of Fig 2 *a* show how the relation between uptake in the two systems is affected by the selectivity of a com petitive inhibitor the concentration of which runs the whole gamut The short line fragment near the corner B_1 is part of the selectivity line of an inhibitor with a selec

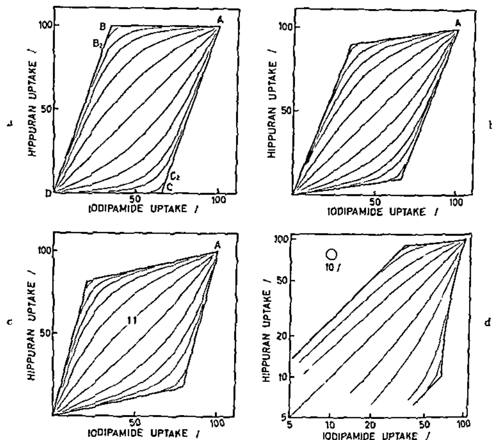


Fig 2 The relative depression of hippuran and iodipamide uptake by inhibitors of different selectivity The graphs inside the parallelogram have been computed according to the following formulae which result from simple Michaelis Menten competition for uptake between an inhibitor and the *test substances*
Hippuran uptake % = in Fig 2*a* 100 F_H in Fig 2 *b—d* 90 $F_H + 10\ F_L$
Iodipamide uptake % = 35 $F_H + 65\ F_L$
The F are free receptor fractions (Barány 1971)
$F_H = 1/(1 + \alpha + [S] \times A_H)$ $F_L = 1/(1 + [S] \times A_L)$
[S] is the concentration of inhibitor A_H and A_L its affinities for the H and the L system respectively and α is zero except in Fig 2 *c* where it is 1 0 The lines within the parallelogram correspond to different ratios A_L / A_H which means inhibitors of different selectivity for the H or the L system Further explanation see text

tivity factor 1000 that is 1000 times more affinity for the L system than for the H system the short line near corner C_1 represents an inhibitor with 1000 times more affinity for H than for L (The lines are not shown in full because they fuse with the others close to A and D) The straight diagonal represents complete unselectivity equal affinity to H and L and counting from the diagonal the different lines represent selectivity factors of $\sqrt{10}$ 10 10 $\sqrt{10}$ 100 and as mentioned 1000 in favour of the one system or the other

Fig 2 *b* represents in exactly the same manner the case where hippuran is not a

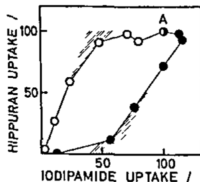


Fig 3

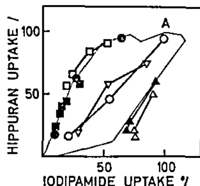


Fig 4

Fig 3 Relative depression of specific uptake of iodipamide and hippuran in slices of rabbit kidney cortex by benzoylglycine (hippurate) and biligrafin (iodipamide) Data of Fig 2 and 3 of Bárány (1972) for 20 and 30 min incubation combined (22—24 slices per point) Filled circles preferential inhibition of the H system by increasing concentrations of benzoylglycine from zero at A to 30 mM Open circles preferential inhibition of the L system by increasing concentrations of biligrafin from 0 1 μM at A to 1 25 mM The hatched areas are explained in the text The outline of this graph returns in most of the following figures

Fig 4 Selectivity of o-iodohippurate ioglycamic acid (Biliastan®) and the non iodinated parent of iodipamide compared with that of benzoylglycine and biligrafin The loop with its apex at A is the same as in Fig 3 thus the left part is generated by biligrafin the right part by benzoylglycine

△ o-iodohippurate 36—240 μM 4 expts 2 rabbits
▲ o iodohippurate 36—240 μM 6 expts 3 rabbits 15 mM acetate
In both these the iodipamide concentration was high 1 μM
□ ioglycamic acid. Single point 100 μM 1 expt Connected points 10—100 μM 4 expts 2 rabbits
■ ioglycamic acid 160—800 μM 2 expts 2 rabbits Slices washed at 0 C after incubation
▽ non iodinated biligrafin 10 μM—1 mM 1 expt
○ non iodinated biligrafin 10 μM—1 mM 2 expts 2 rabbits
● biligrafin as control 10 μM—1 mM 2 expts 2 rabbits
There were 2 slices per point in each separate experiment and the percent uptake figures were averaged

completely specific test substance but where a tracer concentration of hippuran is accumulated to 90 % by the H system and 10 % by the L system This latter figure is probably near the maximum value that could exist in the rabbit kidney and still have escaped detection (Hippuran uptake by the L system has not yet been proved experimentally)

Fig 2 *c* represents the same case as 2 *b* but with the test substance hippuran at such a high chemical concentration that the H system is already half saturated at the starting point A This in reality corresponds to 100 μM hippuran in the medium and can easily be avoided The graph shows that the line of equal affinity to H and L now is not straight but runs a curved course above the (dashed) diagonal With too much iodipamide instead it runs below the diagonal Thus if there is too much of the test substance for one system the affinity of an unknown inhibitor for that system may appear too low

Fig 2 *d* finally is part of 2 *b* drawn on a logarithmic grid This graph conce

the possibility of distinguishing between rather selective competitive inhibitors of different selectivity Experience shows that the absolute error of a percent uptake figure decreases markedly when percent uptake decreases down to moderately low values (see e g Fig 2, 3 and 8 in Barany (1972)) In fact the coefficient of variation tends to be constant down to around 10 % of maximum uptake and quite similar for iodipamide and hippuran Thus the area of uncertainty around any experimental point will be roughly circular and reasonably independent of the position of that point if the graph is plotted on a logarithmic grid Therefore, the separation between the selectivity lines in Fig 2 *d* immediately gives an idea of how difficult it is to distinguish between them experimentally The lines are the same as in Fig 2 *a* except that the 10 $\sqrt{10}$ lines are not shown The maximum distance in the upper *left* hand corner between the *100 1* and *1000 1 lines* is *0 03 log* units Since the ± 10 % coefficient of variation circle (roughly corresponding to a single experiment) shown has a radius of 0 041 log units this shows the high precision necessary to prove that an inhibitor is more specific for the L system than 100 1 Matters are somewhat easier in the lower right hand corner around 10 % hippuran uptake the maximum distance separating the 1 100 and 1 1000 lines is 0 07 log units

The difference between the situation in the 2 corners is due to the difference in selectivity of the 2 test substances hippuran and iodipamide If one had a more specific test substance than iodipamide it would be easier to detect more specific inhibitors of the L system A little could also be gained by choosing a tissue with relatively more of the L system such as the rabbit choroid plexus but this tissue of course is not available in sufficient quantity The liver itself is not suitable since the hepatocyte evidently has transport systems for iodipamide at both ends

Results

1 *Hippurate and biligrafin as inhibitors*

Fig 3 shows the data of Fig 2 and 3 of Barany (1972) plotted in the manner just described In these experiments performed with rabbit kidney cortex slices sodium hippurate was used as inhibitor of the H system and for lack of anything better biligrafin as inhibitor of the L-system The biligrafin anion of course is chemically identical with that of iodipamide but to avoid confusion the *test substance* will be called iodipamide the *inhibitor* biligrafin Similarly the *test substance* will be called hippuran and the *inhibitor* benzoylglycine instead of hippurate

The open symbols represent points obtained with increasing concentrations of biligrafin ranging from 10^{-7} M at A to ~ 10^{-3} M close to the origin The filled symbols represent points obtained with increasing concentrations of benzoylglycine ranging from zero at A to 3×10^{-2} M near the origin The concentration of labelled hippuran was 1.4×10^{-7} M throughout which is about 1000 times below half saturation the concentration of labelled iodipamide was 10^{-7} M which gives about 10 % selfdepression This causes very little error

Except for the apparent stimulation of iodipamide uptake by low concentrations

of benzoylglycine visible as an extension above 100 % in the upper right hand corner which will be discussed the graph shows an evident similarity with the theoretical graphs of imperfectly specific test substances and inhibitors just described It has to be stressed however that one does not know which of the selectivity lines of Fig 2 *a* and *b* that correspond to the lines of Fig 3 In other words we do not yet know how selective for the L and H systems biligrafin or benzoylglycine are By analyzing the biligrafin data in a different manner (Table I Barany 1972) an estimate of the affinity of the substance for the 2 main systems accumulating biligrafin was obtained The affinity for the one was maybe 100 times less than for the other If as is probable the former is the H system the biligrafin part of the boundary in Fig 3 the upper and left parts would correspond to a selectivity line of around 100 1 No similar information for the right hand part of the graph is available Even the estimate of biligrafin selectivity is not reliable since it is based on simplifying assumptions Be that as it may selectivity of any substance can now at least be roughly judged by comparing it with the selectivity of benzoylglycine and biligrafin More specific inhibitors than benzoylglycine and biligrafin would yield distinguishable points mainly in the hatched regions of Fig 3 while less specific inhibitors than the two standard ones will yield points within the boundary

The graph of Fig 3 is applicable only to rabbit kidney cortex There are not enough data for a similar graph referring to choroid plexus or anterior uvea in any species Therefore the present paper is mainly concerned with the rabbit kidney

2 Selectivity of some congeners of hippurate and biligrafin
Iodination

As a rule iodination increases the propensity for biliary excretion at the expense of urinary excretion (for references see Smith 1966) Fig 4 shows however that o iodohippurate used as inhibitor in the usual medium yields points outside the lower right hand corner In a medium with 15 mM acetate the displacement to the right seems to be a little less To some extent this displacement may be due to the overly high iodipamide concentration used in these experiments 15 μM This will partly saturate the L system and may simulate too high an affinity for the H system Anyhow o iodination of benzoylglycine has not increased its affinity for the L system

In iodipamide however iodination has the opposite effect on affinity The non iodinated parent of iodipamide adipic acid di(3-carboxyanilide) was tested in 2 types of experiments In the one it was directly dissolved as any other chemical in the incubation solution (1 expt) In the other (2 expts) it was first dissolved in the solvent of biligrafin and then dilutions made exactly as with biligrafin ampoules Biligrafin ampoule solution was run as a control with tissue from the same animal The results of the control runs were gratifyingly similar to the average biligrafin curve The non halogenated compound in both types of experiment had much less specificity for the liver like system than iodipamide and in fact runs close to the diagonal

The close congener of iodipamide ioglycamic acid (Bilivistan®) was

cording to two protocols The usual one resulted in the open squares slightly left of the biligrafin line (5 expts) In the other 2 experiments the slices after incubation were quickly transferred into ice cold basal incubation solution without test sub stances and inhibitors and then picked up and weighed after 8—20 min The filled squares represent these experiments

The ioglycamic acid points obtained with the usual protocol fall outside the boundary in the region where substances more selective than biligrafin are expected It is possible that bilivistan is in fact such a substance but more experiments are needed to settle the point Anyhow even if it is better it is not completely selective for the L system, since it depressed hippuran uptake down to quite low levels in the experiments of the second kind shown In fact bilivistan can depress hippuran up take in all tissues tested as deeply as does biligrafin

3 *Selectivity of bile and urine*

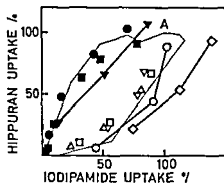
If the transport systems studied in the present experiments in fact are similar to those of the intact liver and kidney then bile and urine might contain inhibitory substances with affinity to the L and H systems Fig 5 shows experiments with bile from the rabbit gall bladder and with rabbit and human urine The animals were those used for the current uptake experiments and the fluids obtained by puncture of the urinary and gall bladders after laparotomy The human urines were passed a few hours earlier

In one experiment the bile from one single rabbit was tested This sample had clearly less specificity for the L system than biligrafin One sample consisting of 3 pooled biles had very much the same selectivity as biligrafin (2 expts) and another sample of 3 pooled biles slightly less selectivity (3 expts) The marked L selectivity of the bile excludes the possibility that the inhibition is due to unspecific cell damage The differences in selectivity between different samples probably are due to that bile is a mixture may contain varying proportions of several substances with different selectivities and that the selectivity of a mixture is intermediate between those of the components

Among the components of the bladder bile bile acids are likely to be responsible for the inhibition Bile salts are the dominant solute in bladder bile and experiments with pure bile acids conjugated as well as unconjugated show that several of them have marked selectivity for the L-system (Barany to be published) Bilirubin glucuronide another possibility has not yet been tested

The experiments with urines showed them to be H selective

In the experiments with human urine dilutions of human and rabbit urine were run simultaneously using the same two control bottles with 2 control slices each Thus the selectivities of both rabbit and human urine would be similarly affected by an error in the uptake figures of the control slices The two rabbit urines and one human urine were all but indistinguishable But one human urine (passed a few hours after a large cup of coffee) deviated far to the right indicating that in dilu tions 1/625 and 1/125 it stimulated the uptake of iodipamide The two slices at



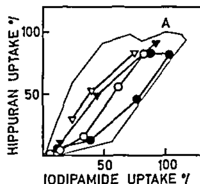


Fig 5

Fig 6

Fig 5 Selectivity of rabbit bladder bile and rabbit and human urine Filled symbol b le open symbols urine
▼ 1 b le dilutions 5 6—5 3 1 expt
○ rabbit urine dil 5 4—5 1 expt
● 3 biles pooled dil 5 6—5 2 expts 2 rabbits
■ 3 biles pooled dil 5 6—5 2 3 expts 3 rabbits
△ rabbit urine dil 5 4—5 1 expt
▽ human urine d l 5 4—5 same expt common control slices
□ rabbit urine d l 5 4—5 2 1 expt
◇ human urine dil 5 4—5 2 same expt. common control slices
There were 2 slices per point in each separate experiment and the percent uptake figures were averaged

Fig 6 Selectivity of probenecid and its higher and lower homologues
○ probenecid 1—625 μM 10 expts 10 rabbits 10 slices/point (20 min data from Fig 8 Barany 1972)
● diethyl homologue 0 1 μM—1 mM 5 expts 5 rabbits 10 slices/point
▽ dibutyl homologue 3— 30 μM 1 expt 2 slices/point
▼ dibutyl homologue 1—100 μM 1 expt 2 slices/point

each concentration agreed closely It therefore seems that the stimulation was real It is similar to the 15 % stimulation of iodipamide uptake by very low concentrations of hippurate seen in Fig 3 of Barany (1972) and visible in Fig 3 of the present paper as the bulge above 100 % iodipamide uptake in the upper right hand corner

These experiments with body fluids speak in favour of the concept that the liver like L-system is concerned with substances mainly excreted by the liver while the H system is concerned with candidates for urinary excretion The presence of the L-system in the kidney is puzzling however Maybe its function is to withdraw certain end products such as *e g* lithocholic acid from too extensive enterohepatic circulation

4 *The selectivity of probenecid and its higher and lower homologues*

Time independence of selectivity

Fig 6 shows the results of experiments with probenecid with its diethyl homologue ethebenecid (Longacid neu ®) and its dibutyl homologue (Longacid®) The data for probenecid are those of Fig 8 in Barany (1972) 20 min incubation

Probenecid itself is rather unspecific with somewhat more affinity for

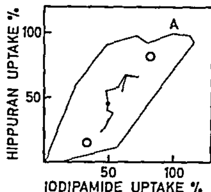


Fig 7

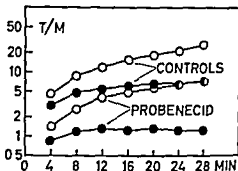


Fig 8

Fig 7 Time independence of selectivity of probenecid The figure shows 2 time series which happen to start at the same point the black dot Running downwards from the dot incubation with 25 μM probenecid for 4—28 min 4 expts 4 rabbits 4 exptl and 4 control slices per point Running upwards from the dot incubation with 10 μM probenecid for 5—40 min Data of Fig 10 in Bárány (1972) 2 expts 2 rabbits 2 exptl and 2 control slices per point The 2 circles are taken from Fig 6 and represent probenecid incubation for 20 min

Fig 8 Time course of uptake of hippuran ● and iodipamide O in the absence and presence of 25 μM probenecid Same experiments as illustrated in Fig 7

system (Barany 1972) This is seen also in the graph the selectivity line is convex towards the abscissa Interestingly enough the diethyl homologue while still much less specific than benzoylglycine is more specific for the H system than probenecid while 2 expts with the dibutyl homologue agree in showing it to be more biligrafin like than probenecid itself

Huang and Lin (1965) have estimated the K for PAH transport into isolated rabbit tubules of probenecid and its diethyl homologue The figures were 0 060 and 0 093 mM Thus the greater selectivity for the H system of the diethyl homologue in the present experiments is not due to greater affinity for that system but to less affinity for the L system There seem to be no similar independent data for the dibutyl homologue in the literature

As shown in Fig 10 of Barany (1972) the degree of inhibition of uptake of both test substances by 10 μM probenecid *decreased* with time from about 50% inhibition at 5 min incubation to about 30% at 25 min During the subsequent 15 min the inhibition again increased to 35—40% These data have now been extended by experiments with 25 μM probenecid Slices were taken out of the medium every 4 min the longest incubation being 28 min Correction was made for the gradual depletion of the medium by uptake At this concentration of probenecid the inhibition increased a little with time In both experiments however the selectivity of probenecid underwent no consistent change with incubation time Fig 7 The 2 meandering lines starting from the black dot (representing the 4 or 5 min points which happen to coincide) evidently do not deviate significantly from the line for probenecid of Fig 6 This line is indicated in Fig 7 by the two open circles between which it runs a practically straight course

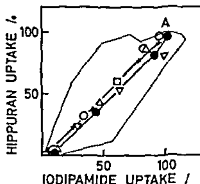


Fig 9 Lack of selectivity of a variety of substances
● Phenol red, 2 μM—2 mM 5 expts 5 rabbits
○ Furosemide 1 μM—1 mM 5 expts 5 rabbits
+ Ethacrynic acid 1 μM—1 mM 5 expts 5 rabbits
▽ Sulfobromophtalein 1 μM—1 mM 4 expts 4 rabbits
△ Iopanoic acid 1—100 μM 4 expts 4 rabbits
□ Phenolpthalein 1 mM 1 expt 2 lices
Every experim nt yielded 2 slices per point

Two similar experiments with kidney cortex from 1 rabbit 4 slices/point were made using 10 mM benzoylglycine which depresses hippuran uptake to a few per cent and iodipamide to about 40 per cent The figures obtained were

Incubation min	4	8	12	16	20	24	28
Iodipamide uptake %	40	39	37	39	42	36	38

In other experiments (not shown) the slices were exposed to 10 or 50 μM of biligrafin 5 min before the labelled test ubstances or as usual dropped into the complete mixture There was no difference in the selectivity of biligrafin (4 ex periments 8 slices/point)

It is interesting to note the difference between the 2 test substances in the time course of uptake Fig 8 shows the data of the 25 μM probenecid experiment The main fact is similar to that of the previous 10 μM probenecid experiment the slice/medium ratio for hippuran levels off at a time when simultaneous uptake of iodipamide still gives rising slice/medium ratios In the calculation of these curves of Fig 8 the unspecific slice/medium ratio has been assumed to be the same as at 20 min incubation 0 91 for hippuran and 0 64 for iodipamide Since with very short incubation this ratio might be a little less the very first points of the curves may be a little too low and the levelling off especially for the inhibited hippuran slices even more marked

5 The lack of selectivity of phenolphthalein phenolsulfonphthalein sulfobromo phthalein iopanoic acid ethacrynic acid and furosemide

Phenol red phenolsulfonphthalein is extensively used in kidney physiology and BSP sulfobromophthalein in liver physiology Fig 9 shows the surprising lack of specificity of these two compounds when used as inhibitors Phenolphthalein the carl analogue of phenol red also is quite un elective

TABLE I Effect of cephaloridine in the medium on the simultaneous uptake of iodipamide and hippuran into rabbit tissues

Expt no	Medium conc mM	Percent of uptake in control slices						Number of independently processed pieces of		
		Hippuran			Iodipamide					
		Kidney cortex	Choroid plexus	Anterior uvea	Kidney cortex	Choroid plexus	Anterior uvea	Kidney cortex	Choroid plexus	Anterior uvea
828	3	73	31	38	75	52	54	4	2	2
829	3	61	42	29	77	58	42	4	2	1
829	10	28	19	12	55	26	27	4	2	2
816	10	22	5	7	41	12	15	2	1	1

Also the 2 diuretics ethacrynic acid (Edecrina®) and furosemide (Lasix®) and the cholecystographic agent iopanoic acid (Bilijodon®) yield points very close to those of BSP and phenol red

It is evident from these experiments that the relative affinity to the H and L-systems (and presumably the kidney or liver itself) is not what makes BSP a test substance for the liver and phenol red one for the kidney furosemide and ethacrynic acid drugs used for their action on the kidney and iopanoic acid a cholecystographic agent Affinity is but one requirement for active uptake and subsequent actions metabolism or secretion

6 *Experiments with cephaloridine*

A large dose of cephaloridine damages the proximal tubules in several species *In* the rabbit 24—96 h after 200 mg/kg there is extensive proximal tubular necrosis while 50 mg/kg is not nephrotoxic (Atkinson *et al* 1966 Welles *et al* 1965) The damage can be prevented by probenecid (Child and Dodds 1966) and the authors suggest that the drug acts after intracellular accumulation in the tubules This suggestion is supported by recent findings of Tune (1972) who found that 30 min after a subcutaneous injection the antibiotic was present in high concentration in the renal cortical tissue of rabbits Both the accumulation and the damage could be prevented by probenecid given before the cephaloridine Very high doses of benzylpenicillin likewise prevented the accumulation Probenecid inhibits both the H and L systems but whether benzylpenicillin also has some affinity for the latter is not yet known Anyhow it is clear that the damage caused by the drug is due to active uptake into cells possessing an organic anion transport system inhibitable by probenecid

In vitro tested with the present technique cephaloridine (Kefspor® Lilly) at 1 mM caused marginal depression of uptake At 3 and 10 mM however there was a clearcut effect (Table I) The uptake of both test substances was inhibited and the inhibition was clearly visible in all three tissues tested if anything it was more pronounced in plexus and uvea than in renal cortex Cephaloridine seems to be somewhat H selective

TABLE II Effect of 200 mg/kg cephaloridine s.c. on the simultaneous uptake of hippuran and iodipamide into rabbit tissues. One animal was tested at 24 h, 4 at 48 h after the treatment. Each animal contributed 6–12 pieces of kidney cortex, 3–6 of uvea and 3 of choroid plexus besides the totally inhibited 2–4, 1–2 and 1 piece respectively. Means ± S.E. Statistical unit: animals

	Tissue/Medium ratio T/M				T/M Hippuran	
	Hippuran		Iodipamide		T/M Iodipamide	
	Treated	Control	Treated	Control	Treated	Control
Choroid plexus	1.71 ± 0.22	1.80 ± 0.12	10.18 ± 0.32	11.45 ± 0.43	0.167 ± 0.01	0.159 ± 0.011
Anterior uvea	4.72 ± 0.53	6.62 ± 0.64	7.45 ± 0.66	8.89 ± 0.44	0.649 ± 0.076	0.[illegible]43 ± 0.049
Kidney cortex	1.92 ± 0.21	7.72 ± 0.51	5.23 ± 0.41	14.52 ± 1.05	0.356 ± 0.018	0.547 ± 0.070
Kidney cortex controls from Methods						
bottle 1 (n = 34)		7.59 ± 0.30		14.43 ± 0.71		0.543 ± 0.017
bottle 2 (n = 31)		7.72 ± 0.25		15.33 ± 0.89		0.534 ± 0.019

Systemic cephaloridine 50 mg/kg s.c. tested after 48 h gave no significant depression (1 treated, 3 controls). 100 mg/kg at 48 hours caused a barely significant (2 S.D.) 51 % depression of hippuran and a not significant 41 % depression of iodipamide (1 treated, 1 control). Thus a dose of 200 mg/kg was decided upon: it caused clearcut damage at 24 and 48 h. The figures at 24 h were so similar to the others that the data were pooled for the 2 time intervals. Table II shows the results obtained with 5 treated and 5 control animals. The controls agreed very well with the large group of controls (not including the present ones) analyzed in the Methods section. In the injected animals the kidney cortex was markedly lighter in colour and there was a significant depression of the uptake of both test substances into the kidney. The uptake into choroid plexus or uvea was, however, but little depressed.

In the kidney slices the uptake of hippuran was more depressed than that of iodipamide. The difference in the ratio (T/M hippuran)/(T/M iodipamide) is significant at $P < 0.001$ ($t = 7.2$, d.f. = 8). This difference has interesting implications. If both transport systems were located in the same cells and present in roughly equal proportions in all the cells, extensive necrosis would cause equal loss of uptake *in both* systems. This was not observed. *The most probable explanation is that the* cell population harbouring the two transport systems is heterogenous, and that cells which are killed contain disproportionately much H- or little L-system. Cephaloridine does not kill indiscriminately: in the mouse Atkinson *et al.* (1966) found that the most sensitive tubules lay immediately beneath the capsule and with more severe involvement the tubular necrosis spread toward the cortico-medullary junction, while in the rabbit Silverblatt *et al.* (1970) found that mainly the second segment of the proximal tubule was damaged.

As mentioned, there was very little if any effect on the uptake in anterior uvea or choroid plexus. Table I shows that both the H- and the L-system of uvea and plexus do have affinity for the drug *in vitro*. The most probable explanation for t[illegible]

of depression by systemic treatment therefore is that the transport systems of these tissues do not accumulate cephaloridine when it is offered from the blood This gives a clue to the direction of transport of the L-system in these tissues

In the plexus (Pappenheimer Heisey and Jordan 1961) and uvea (Forbes and Becker 1960) transport by the H system *in vivo* is towards the blood In the kidney transport is in the opposite direction the H system pump is located at the peri tubular cell border adjacent to the blood From analogy one would then expect the H pump in plexus and uvea to be located at the cell border not adjacent to the blood and therefore not accessible from the blood This would explain why cephaloridine is not taken up by the H system of these tissues If now the L-system were pumping in the direction blood tissue in these tissues one would have expected uptake of cephaloridine and damage since cephaloridine has affinity to the L-system too *The fact that no damage occurred indicates* that the L-system in these tissues moves acids in the same direction as the hippurate system out of the eye and out of the cerebrospinal fluid

Discussion

A large body of knowledge exists concerning the excretion of organic acids by the kidney and the liver (see Despopoulos 1965 Schanker 1968) The present experiments concern a related problem but it cannot be overemphasized that competition experiments do not allow conclusions about the actual uptake and further [illegible] of the inhibitory compounds

If one has a composite uptake system and a competition experiment is made with only one test substance the affinity of an inhibitor to the several components of the uptake system the selectivity of the inhibitor can be analyzed by curve fitting or by solution of the appropriate equations The mathematics is shown in Barany (1971) and some results of such curve fitting in Barany (1972) The method requires that the inhibition is studied over the full range which may imply a large number of inhibitor concentrations Also input data of high precision are needed On the other hand real affinity values are obtained

If two different test substances with different specificities are available as in the present experiments the much less laborious method described here can be used for judging the specificity of the inhibitor As used here it does not give real affinity values but it could be extended to do so If it then will turn out to be less fastidious with respect to the input data has not been checked as yet

It has been blandly assumed that all inhibitors were competitive This is a likely assumption for most of the substances studied but very hard to prove without really specific test substances or inhibitors A composite system can hide behind a reasonably good fit in a Lineweaver Burk plot and the conclusion derived from the plot can then be completely misleading (Christensen 1966) In the rabbit renal cortex we do have at least 2 systems It has incidentally been a great boon to kidney physiology that the dog kidney almost completely lacks the L-system (Berndt and Mudge 1968 Barany 1972)

The unsofisticated slice technique as used here is convenient but represents a complex situation (Bojesen and Leyssac 1965) and conclusions will have to be tentative

For the H system in rabbits the most advanced technique has confirmed that the only active pump is located at the peritubular cell border and that the luminal side of the cells is passive (Tune Burg and Patlak 1969) How far is this true also for the L-system? Since for instance phenol red does not concentrate in the lumen of mammalian tubules (Forster and Copenhaver 1956) and since we have seen that phenol red has affinity to both the H and the L systems most probably the L system also causes no concentration in the lumen

For the H system it is accepted that the site of competition is at the basal end of the cells This is not necessarily so for the L system even if the present experiments showing time independence of the selectivity of probenecid are most easily explained that way On the other hand the possibility exists that some substances compete both for the first step of uptake and for intracellular binding while others compete for only one of the two

Could the L system be a reabsorptive system located at the luminal side? This seems improbable because of the collapsed tubular lumens in the slice One would expect a marked difference in the time course of concentration of for instance probenecid between the peritubular space and the intraluminal (collapsed) one This should lead to time dependent selectivity

The selectivity graphs are a conscious oversimplification There is some evidence that the I system is composite (Barany 1972) Since there is no reason why its components should have similar affinities to all inhibitors the selectivity graphs should in fact have been three or more dimensional At the present state of ignorance however the two-dimensional ones are all that can be constructed

The apparent stimulation of iodipamide uptake by a human urine (Fig 5) and the probable stimulation by very low concentrations of hippurate is a disturbing phenomenon Besides with hippurate and ethebenecid (Fig 6) it has also been observed in a few experiments with low concentrations of picolinic nicotinic and some other heterocyclic acids It is well known that run out of dyes or test substances from slices or isolated tubules of mammalian kidneys is stimulated by low and inhibited by high concentrations of competitors (Farah Frazer and Stoffel 1963 Huang and Lin 1965) what we see here would seem to be the opposite phenomenon A substance which shows this phenomenon may appear to have a falsely high selectivity for the H system since the substance so to speak prevents itself from depressing iodipamide uptake properly The phenomenon evidently has to be analyzed further

The differences in selectivity between the probenecid homologues offer interesting possibilities The lipid solubility of probenecid has made it a useful inhibitor for outwards directed transport of acid metabolites from the brain (Neff Tozer and Brodie 1967 Werdinius 1967 Ashcroft Dow and Moir 1968 and Tamarkin Goodwin and Axelrod 1970 to mention but a few) Its low specificity makes it a ca

all, however Perhaps the higher and lower homologues tested here or even more different one would be useful as more selective inhibitors of the different transport systems *in vivo* A differentiated inhibition by probenecid homologues was in fact already envisaged by Bruckschen (1955) but the matter seems not to have been followed up and the probenecid homologues have disappeared from clinical use It could well be that the remarkably large effects observed in humans by Tamarkin *et al* (1970) with enormous doses of probenecid are due to inhibition of the L system and would have been more easily achieved with a higher homologue

While the direction of transport of the L system cannot be said to be definitely established the cephaloridine experiments indicate that it is the same as the direction of transport of the H system If this is really so the L system in the uvea and choroid plexus may be a liver like guard system protecting the eye and the brain from metabolites destined for excretion mainly by the hepatic route

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Acta physiol scand 1973 88 430—432
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Large Vesicles of the Adrenergic Nerves of the Rabbit Uterus

By

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Considerable evidence has accumulated that nerves containing noradrenaline as transmitter are characterized by a predominance of small granular vesicles (300—600 Å) the dense core of which can be specifically demonstrated using $KMnO_4$ fixation It is also generally accepted that the cholinergic terminals contain predominantly small agranular vesicles A few large (700—1200 Å) granular vesicles are present in both types of axons probably containing catecholamine in the adrenergic axons and some as yet unknown substance in the cholinergic axons Since this basic division of the peripheral autonomic nerves and axon terminals into two further types of terminals have been described The fine structural features of the non adrenergic inhibitory nerves were recently characterized (Robinson *et al* 1971) It was shown hat the axon profiles of the toad lung responsible for the mediation of the inhibitory impulses contained predominantly large granular vesicles (600—2000 Å) unlike the ordinary large granular vesicles of the adrenergic and cholinergic nerves (700—1200 Å)

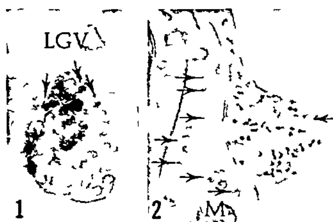
The present study will describe the axons filling the morphological criteria of the inhibitory nonadrenergic nerves in the rabbit myometrium

Adult female albino rabbits were used 4 rabbits received daily injections of 10 μg/kg of 17 β-estradiol for 14 days To induce the degeration of the adrenergic nerve terminals 4 other rabbits received 75 mg/kg of 6-OH-dopamine in single i v injection. The specimens were prepared under pentobarbital anesthesia Small pieces of uterus and oviduct were fixed in icecold 3 % $KMnO_4$ for 60 min dehydrated in ethanol after a short rinse and embedded in EPON Ultrathin sections were cut on an LBK utratome and studied with a Philips 300 electron microscope operated at 40—60 kV

In addition to the two ordinary types of autonomic axons adrenergic characterized by the predominance of small granular vesicles and cholinergic (or non adrenergic) characterized by the predominance of small agranular synaptic vesicles —a third type of axon profiles was regularly found The main feature of the third type of axon was the presence of variable amounts of large vesicles (LV) the diameter of which ranged from 600—2200 Å To avoid confusion the LV are not named as either granular or agranular in the present study The internal structure

Fig 1 Solitary axon profile containing both large granular vesicles (LGV) and large agranular vesicles Note the reticular substructure of the agranular vesicles ×25 000

Fig 2 Two axons in the myometrium of the rabbit One is filled with small granular vesicles typical for the adrenergic axons while the other one contains only large agranular vesicles (arrows) M mitochondria ×25 000



of the vesicles consisted of fine reticulum the density of which varied only very little from vesicle to vesicle The electron opacity of the reticulum and the vesicular matrix was mostly about the same as that of the surrounding axoplasm Occasionally distinctly more electron opaque vesicles were also found but even they were still far from the density of the noradrenaline storing small and large granular vesicles of the adrenergic nerve endings of the neighbouring axons Thus it would be misleading to name the large vesicles granular' Treatment with 6 OH dopamine did not destroy the axonprofiles containing LV while all the adrenergic axons degenerated

Treatment with large doses of 17 β-estradiol increased the amount of LV in the adrenergic axons (For details see Hervonen *et al* 1972) The frequency of axon profiles containing solely LV also seemed to increase However the network formed by the axons containing solely large vesicles seemed to be very sparse Searching for this type of axon required extensive serial sectioning even in areas with a dense network of ordinary autonomic nerves Less than 1 % of the total count of axon profiles could be placed in the third group

An intermediate form of axon profiles between the ordinary adrenergic ones and the type containing solely LV was found more frequently than the pure third type These endings contained a few large granular vesicles which are typical of the ordinary adrenergic axons after $KMnO_4$ fixation

Both pharmacological and electrophysiological evidence has been presented for the existence of non adrenergic inhibitory nerves The fine structural features of the non adrenergic inhibitory nerves have been recently described by several investigators (Bennett and Cobb 1969 Robinson *et al* 1971) These axons were characterized by the presence of predominantly large vesicles (600—2000 Å) containing homogenous moderately electron opaque material after glutaraldehyde fixation In the present study $KMnO_4$ fixation (Hokfelt 1969) was used in order



Fig 3 Larger magnification of an axon containing only large agranular vesicles (LV) The internal structure of the vesicles is of the same electron opacity than the surrounding axoplasm ×50 000

to differentiate the adrenergic and non adrenergic axons After this fixation the *content of the large vesicles was mostly reticular and no dense grains could be found* Thus if the large vesicles store some transmitter it is evidently not noradrenaline Furthermore treatment with 6 OH dopamine which induced the degeneration of all the adrenergic axons of both oviduct and uterus did not alter the fine structure of the profiles filled with the large vesicles Nerves containing purely LV have not been described after permanganate fixation Treatment with 17 β estradiol caused an increase in the amount of the axon profiles containing LV as well as other changes described previously by Hervonen *et al* (1972) Estrogen causes an increase in the intra axonal smooth endoplasmic organelles in the adrenergic axons too The LV and other forms of the intra axonal membraneous structure might represent a potential capacity for transmitter storage Thus the axons containing solely LV need not necessarily be a third type of axon basically different from the ordinary autonomic axons *It also seems reasonable to assume that the variations in the* amount of smooth endoplasmic components of the axoplasm especially after estrogen treatment could lead to accumulation of LV in certain parts of the axons Thus the LV might be a sign of a certain functional or metabolic state prevailing in the neuron

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Acta physiol scand 1973 88 433—439
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Vomiting and Reflex Vagal Relaxation of the Stomach Elicited from Heart Receptors in the Cat

By

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Abstract

Abrahamsson, H and P Thorén *Vomiting and reflex vagal relaxation of the stomach elicited from heart receptors in the cat* Acta physiol scand 1973 *88* 433—439

The effect of afferent stimulation of the vagal cardiac nerves on gastric motility heart rate and arterial blood pressure was studied in cats Electric stimulation of the right cardiac nerve elicited beside bradycardia and hypotension a prompt and pronounced gastric relaxation and after a latency somatomotor vomiting movements —Frequency response curves showed maximal circulatory effects already around 6 Hz while the curve for gastric relaxatory response was less steep Frequent retching was observed at stimulations of 10 Hz or more —The gastric relaxation upon afferent cardiac nerve stimulation was abolished by sectioning the right vagus caudad to the cardiac nerve and the left vagus in the neck showing that vagal efferents mediated the gastric relaxation. The gastric response persisted after atropine and guanethidine and was mimicked by reflex activation of the vagal relaxatory' fibres to the stomach by esophageal distension. Intrapericardial nicotine and coronary artery occlusion also activated the described gastric reflex —It is concluded that vomiting can be elicited by thin vagal cardiac afferents probably of ventricular origin Gastric relaxation mediated by the vagal non adrenergic relaxatory fibres constitute part of this vomiting response as when vomiting is induced by apomorphine

It was recently shown (Abrahamsson and Thorén 1972) that activation of non myelinated vagal afferents emanating from the left ventricle of the heart can besides reflex bradycardia and arterial hypotension also elicit a marked reflex relaxation of the stomach This gastric relaxation can be produced by electrical excitation of the vagal cardiac afferents The same response can be elicited by mechanical or chemical stimulation of the heart receptors either by distension or ischemic bulging of the left ventricle by stroking along the left ventricular epicardium or by veratrum alkaloids and nicotine The possibility was suggested that this gastric response could be part of a vomiting response pattern

Studying the effects of the emetic drug apomorphine on gastric motility Abrahamsson Jansson and Martinson (1973) found that vomiting produced by this drug was regularly associated with a pronounced gastric relaxation mediated by

vagal non adrenergic relaxatory fibres (Martinson 1965) The present experiments were designed to see whether the reflex gastric relaxation elicited by the above mentioned heart receptor stimulation, is also part of a vomiting response and to explore the efferent nervous mechanisms to the stomach

Material and Methods

Experiments were performed on 16 cats (weight 2 4—4 6 kg) deprived of food for 24—36 h before the experiment 12 animals were anesthetized with chloralose 40—50 mg/kg b w and 4 expts were performed on unanesthetized decerebrate cats Ischemic decerebration was performed during ether anesthesia as described elsewhere (Abrahamsson Jansson and Martinson 1973)

After tracheotomy the thorax was opened by a right intercostal incision with the animal on artificial ventilation The pericardium was widely opened with the edges sewn to the chest wall After division of the azygos vein the right main cardiac vagal nerve was cut in 12 expts and the central end was placed on a bipolar electrode

Arterial blood pressure was measured from a subclavian artery by a Statham pressure transducer (P23AC) connected to a Grass Polygraph 7A recorder while heart rate was monitored by a tachograph triggered by the arterial pressure pulse

Gastric motility was recorded as volume changes at low intragastric pressures (4—8 cm H_2O) by means of a large rubber balloon introduced through the esophagus (Jansson 1969) Via a wide waterfilled volume reservoir the balloon was then connected either to a float recorder writing on a kymograph or to a small reservoir connected to a Grass FT 03 C force displacement transducer operating a Rikadenki writer

Experimental procedures In the decerebrate animals the right cardiac nerve was electrically stimulated in afferent direction at varying frequencies (2—3 ms 4—6 V) and besides measuring changes in blood pressure heart rate and gastric volume the animals were observed for retching They were then placed on their side since supine cats vomit only with difficulty (see e g Alvarez 1948 p 488)

In the chloralose anesthetized cats the receptor afferents were stimulated either electrically (0 5—50 Hz, 1—2 ms 4 V) or when left intact mechanically by different means e g coronary occlusion and chemically by nicotine (20—100 µg) in saline injected into the pericardial cavity —Distension of the esophagus was performed by inflating 10—16 ml air into a small rubber balloon placed in its thoracic part

To examine the efferent nervous pathway to the stomach the left vagus was cut in the neck and the right one just caudad to the entrance of the stimulated right cardiac nerve To block the cholinergic excitatory fibres to the stomach atropine (Atropine sulphate Merck) 0 5 mg/kg b w was given i v in 7 cats and guanethidine (Ismelin® CIBA) was used as antiadrenergic substance in i v doses of 3—5 mg/kg b w in 4 cats

Results

Retching circulatory and gastric responses upon cardiac nerve stimulation Afferent cardiac nerve stimulation was performed in unanesthetized decerebrate cats while changes in gastric motility heart rate and arterial blood pressure were recorded and the animals observed for retching Fig 1 is from such an experiment where the right cardiac nerve was stimulated electrically with 20 Hz 2 ms and 4 V With a latency of only few seconds a profound gastric relaxation, a blood pressure fall and a marked bradycardia ensues After another 40 s of stimulation retching starts with forceful contraction of the abdominal muscles which causes a rapid decrease of gastric volume However as soon as retching movements cease the relaxed stomach immediately returns to its enhanced volume to regain its prestimulatory volume only slowly The latency from onset of stimulation to the beginning of retching varied from 30 to 90 s

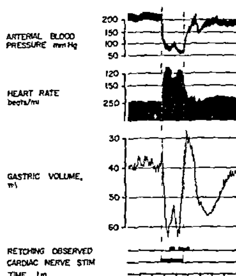


Fig 1 Cat 3 8 kg unanesthetized decerebrate Circulatory and gastric changes to afferent stimulation of the right cardiac nerve (20 Hz 2 ms 4 V) Note the initial gastric relaxation with short latency (around 5 s) After about 40 s of continued stimulation retching is observed with simultaneous phasic decreases in gastric volume After the retching has ended the stomach returns to its widened relaxed state and only slowly returns towards control volume

In 4 unanesthetized cats where the cardiac nerve was stimulated at supramaximal strength but with varying frequencies it was observed that retching occurred only occasionally at frequencies below 10 Hz but in 6 of 10 stimulations at 10 Hz and in 13 of 18 stimulations at 20 Hz or more For comparison frequency response characteristics of the blood pressure fall and of the gastric volume increase upon afferent stimulation of the right cardiac nerve were studied in four anesthetized cats Fig 2 clearly shows that the circulatory response obtained maximal values already at about 6 Hz while the gastric relaxatory response was maximal first above 20 Hz In the low frequency range below 6 Hz there was a large difference between the circulatory and gastric response

Analysis of the efferent nervous pathway to the stomach As illustrated in Fig 3 the reflex gastric relaxation elicited by afferent cardiac nerve stimulation persisted after atropine blockade of the cholinergic excitatory fibres to the stomach although the gastric control volume was increased by this drug Such gastric relaxation was obtained after atropine when the cardiac receptors or afferents were activated with electric stimulation (5 expts) chemical stimulation by nicotine intrapericardially

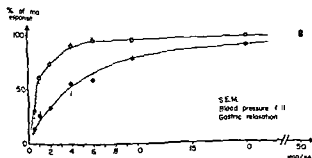


Fig 2 The frequency response curve for the reflex fall in blood pressure and the reflex gastric relaxation expressed as per cent of maximal effects when the right cardiac vagal nerve is stimulated in the afferent direction with increasing frequencies at 4 V and 1—2 ms The diagram is based on 31 stimulations in 4 cats.

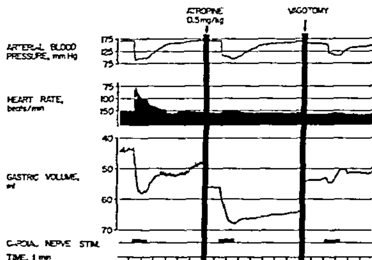


Fig 3 Cat 4 1 kg Chloralose The effect of afferent stimulation (20 Hz, 2 ms 4 V) of the right cardiac nerve on blood pressure heart rate and gastric volume Note that both the gastric relaxation and blood pressure fall persist after injection of atropine (0.5 mg/kg i.v.) but the heart rate changes are greatly reduced (middle panel) After section of the right vagal nerve caudad to the stimulated cardiac nerve and the left vagus nerve in the neck (right panel) the stimulation still produces marked blood pressure fall but no clear gastric relaxation.

(5 expts) and by coronary artery occlusion (2 expts) The reflex gastric relaxation also persisted after the antiadrenergic drug guanethidine had been given (Fig 4) whether induced by electrical stimulation of the right cardiac nerve (2 expts), by *nicotine intrapericardially (4 expts.) or by coronary occlusion (2 expts)*

To see whether the vagal nerves contained the efferent link for the reflex from the heart to the stomach the left vagal nerve was cut in the neck and the right one caudad to the entrance of the stimulated cardiac nerve Fig 3 (right part) shows that the atropine-resistant gastric relaxation elicited by afferent cardiac nerve stimulation is essentially abolished after the mentioned type of vagal section while the circulatory responses to cardiac nerve stimulation can still be seen Thus the

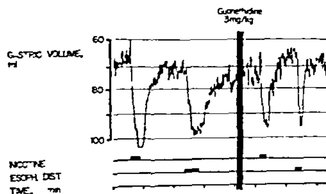


Fig 4 Cat 3 0 kg Chloralose. Reflex relaxation of the stomach elicited by nicotine (60 μg) intrapericardially and by distension of the esophagus before and after administration of the antiadrenergic *drug guanethidine 3 mg/kg*

described reflex gastric relaxatory response appears to be mediated *via* vagal efferent fibres

The above results indicate that activation of vagal cardiac afferents produces a reflex activation of the vagal non adrenergic relaxatory fibres to the stomach This conclusion is also supported by the fact that the reflex gastric relaxation elicited from the heart closely resembles that produced by esophageal distension (Fig 4) which is known to reflexly activate the vagal relaxatory fibres to the stomach (Abrahamsson and Jansson 1969)

To elucidate a possible contribution to the described reflex response of extra vagal adrenergic fibres to the stomach vagotomy was performed as described above Afferent stimulation of the right cardiac nerve had then no inhibitory effect on the excitatory gastric response as induced by an efferent graded stimulation of the left vagal nerve (4—6 Hz, 2 ms 6 V) This means (*cf* Jansson and Martinson 1966) that splanchnic adrenergic fibres are probably not of any importance for the gastric relaxation elicited by afferent electrical stimulation of vagal cardiac fibres

Discussion

The present study has shown that afferent stimulation of the vagal cardiac nerve can induce vomiting in the cat regularly associated with a reflex gastric relaxation which shows the same general characteristics as that seen when vomiting is induced by a central acting emetic agent (Abrahamsson Jansson and Martinson 1973) The somatomotor vomiting movements in response to cardiac receptor stimulation are clearly evident only in unanesthetized cats This indicates that the reflex gastric relaxation elicited by stimulation of cardiac receptors with unmyelinated vagal afferents (Abrahamsson and Thoren 1972) is part of a vomiting reflex where the somatomotor component is easily suppressed by anesthesia

Comparatively high frequency stimulations (10 Hz or more) of the cardiac nerves that have already evoked maximal circulatory changes were required to induce a vomiting pattern including both retching and reflex gastric relaxation at lower frequencies only gastric relaxation occurred A comparison between frequency response characteristics for circulatory and gastric relaxatory effects showed marked differences The circulatory response was almost maximal already around 6 Hz while the less steep frequency response curve for the gastric relaxation reached its maximum first around 20 Hz This difference in the two reflex responses is likely to reflect differences in the central organisation of the reflex pathways In fact the results suggest that the cardiac receptors are primarily engaged in circulatory regulation (*cf* Öberg and Thoren 1972 b) First at increasing receptor stimulation the central emetic mechanism appears to become gradually activated initially resulting in pronounced gastric relaxation and eventually in a complete vomiting response

The described elicitation of emesis from cardiac receptors explain some confusing clinical conditions In man coronary infarction is sometimes complicated by bradycardia (Adgey *et al* 1968) and these patients often suffer from severe nausea and vomiting Increased activity in cardiac receptors can perhaps be the cause of these

symptoms since these receptors are often intensely activated by the myocardial bulging caused by coronary occlusion (Thorén 1972)

Nausea and vomiting are further wellknown prodromal phenomena in vaso-vagal syncope in man Evidence has been presented that vaso vagal reactions can be precipitated by increased activity in left ventricular receptors e g when left ventricular filling becomes very poor (Öberg and Thorén 1972 a) Barclay (1936) described a marked relaxation of the stomach on X ray in standing patients which were just about to faint and accordingly this effect could perhaps be due to a reflex from heart receptors

Nicotine intrapericardially which in our experiments evoked a powerful reflex gastric relaxation did not produce vomiting in unanesthetized dogs (Sleight 1964), but sometimes the dogs showed hyperpnea and salivation which are prodromata in the vomiting act. The failure to produce definite vomiting in this way is probably due to the short time (only 30—60 s) of increased left ventricular receptor activity obtained when nicotine is injected intrapericardially (Öberg and Thorén 1972 b)

Administration of veratrum alkaloids, which activate the same type of left ventricular receptors (Öberg and Thorén 1972 b) is also often accompanied by disturbances like nausea and vomiting These effects have been ascribed to activation of receptors in the nodose ganglion (Borison and Fairbanks 1952) but probably cardiac receptors are the main source also in this respect Digitalis alkaloid which have an emetic effect thought to be partly mediated through some peripheral receptors (see Wang 1965) also activate the left ventricular receptors (Öberg and Thorén 1972 b) Therefore it is possible that at least part of the nausea and vomiting seen after digitalis adminstration is due to increased cardiac receptor activity

The present analysis further showed that the reflex gastric relaxatory response to stimulation of cardiac receptors was not mediated by cholinergic or adrenergic efferent pathways On the other hand it was clearly mediated by the vagal nerves strongly suggesting that the vagal non adrenergic relaxatory fibres to the stomach (Martinson 1965) form the efferent link of the reflex This conclusion is also supported by the observation that the gastric relaxatory response to the heart receptor stimulation exhibited the same general characteristics as the reflex gastric response to esophageal distension which is known to be mediated by the vagal relaxatory fibres to the stomach (Abrahamsson and Jansson 1969) Splanchnic adrenergic fibres which can also suppress gastric motility were probably of minor importance for the recorded gastric reflex response since afferent stimulation of the vagal cardiac nerve had no effect on a vagally induced gastric excitatory motor activity (cf Jansson and Martinson 1966) produced by direct graded stimulation of the cut vagal nerves

The described vomiting response pattern induced from heart receptor afferents is closely similar to that evoked by the central acting emetic drug apomorphine (Abrahamsson Jansson and Martinson 1973) Apomorphine in smaller doses induces gastric relaxation due to activation of the vagal relaxatory fibres In larger doses this drug induces initial gastric relaxation and then vomiting with a latency

of about 1 5 min or more Thus, when vomiting is produced by apomorphine there is a marked difference in the dose required to elicit gastric relaxation and that producing a complete vomiting pattern Such a threshold difference for elicitation of gastric relaxation and vomiting is also evident when the vomiting pattern is reflexly produced by stimulation of the cardiac vagal afferents

Several studies have shown that there is a pronounced relaxation of the upper part of the stomach during vomiting when elicited by apomorphine (Cannon 1898 Hesse 1913) which has a central action (see Wang 1965) or *via* autonomic nervous afferents (see *e g* Cannon 1911) The present results together with the study of effects of apomorphine (Abrahamsson Jansson and Martinson 1973) indicate that a purposeful pronounced gastric relaxation mediated by the vagal relaxatory fibres to the corpus fundus of the stomach always accompanies or preceds the vomiting response however induced

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The Formation of ^{14}C-Histamine from Orally Administered ^{14}C-l-Histidine in Pigs

By

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Abstract

ELIASSEN K A *The formation of ^{14}C histamine from orally administered ^{14}C l histidine in pigs* Acta physiol scand 1973 *88* 440—445

The object of the present study has been to examine to what extent dietary L-histidine is converted to histamine in pigs a species which excretes large amounts of histamine in the urine. During the first 24 h after oral administration of ^{14}C L-histidine only about 1/10 to 1/3 of the administered radioactivity was recovered in the urine Free ^{14}C-histamine accounted for about 1 % *of the urinary radioactivity while the values for* ^{14}C 1 4 *methylhistamine* and ^{14}C-1 4 methylimidazoleacetic acid were about 0 5 and 40 % respectively The values for ^{14}C-histamine may be overestimated but the high values for the methylated ^{14}C-metabolites of histamine indicate that 4—20 % of orally administered ^{14}C-L-histidine is converted to Hi. This is far superior to what has been found in other species. Oral administration of 10 g L-histidine monohydrochloride to pig resulted in the excretion of a much smaller fraction as histamine and as methylated metabolites of histamine than would be expected from the experiments with ^{14}C-L-histidine

L-histidine is the only established precursor of histamine *In vivo* formation of histamine from L-histidine has been demonstrated in most common laboratory animals (Schayer 1952 1956 and White 1960) In man however the demonstration of histamine formation *in vivo* has met with difficulties. Thus neither parenteral nor oral administration of small doses of L-histidine resulted in increased histamine formation as judged from urine analyses By increasing the dose of L-histidine to 5—10 g daily some authors (Irvine Duthie and Waton 1959 Oats Marsh and Sjoerdsma 1962 and Sjaastad 1966) have found increased urinary excretion of free histamine whereas others (Duner Liljedahl and Pernow 1961 and Granerus 1968) found no change in either urinary free histamine or in histamine metabolites *In vitro* however it has been demonstrated that histamine can be formed from l histidine in all examined species

It has previously been demonstrated that pigs excrete large amounts of free histamine with the urine compared to other species (Eliassen 1971 b) It therefore seemed possible that a substantial fraction of dietary L-histidine is converted to

histamine in this species If so pigs might be well suited to demonstrate the relation ship between the levels of dietary L histidine and the histamine formation The ob ject of the present study has therefore been to examine to what extent small amounts of orally fed L-histidine are converted to histamine

In the present study urinary histamine is used as an indicator of the *in vivo* forma tion of the substance However, histamine from any source will be subjected to enzymatic inactivation in the tissues and only a small fraction will reach the urine unchanged In order to obtain more complete information as to the formation of histamine from orally administered histidine two histamine metabolites 1 4 methyl histamine and 1 4 methylimidazoleacetic acid were therefore estimated in addition to ^{14}C histamine in the present study

Abbreviations
H_1 histamine 4(5) (β aminoethyl)imidazole 1 4 MeHi 1 4 methyl histamine 1 methyl 4 (β aminoethyl)imidazole 1 4 MeImAA 1 4 methylimidazoleacetic acid 1 4 methyl imidazole 4 acetic acid

Materials and Methods

4 castrated male pigs of the Norwegian Land swine breed were used The pigs were housed in metabolism cages which permitted separate collection of urine and feces

Sufficient HCl to bring the pH of the urine below 2 was added to the collection bottles which were standing in ice The pigs were fed concentrates twice a day and water *ad libitum* In contrast to previous experiments (Eliassen 1971 b) the food did not contain antibiotics

Administration of ^{14}C labelled L-Histidine
About 50 μCi ^{14}C L-histidine purified on a Dowex 50 W column (Kahlson Rosengren and Thunberg 1963) and dissolved in 200 ml 3 % NaCl was given by way of a stomach tube In 3 expts ^{14}C solution was given 1—4 h before the afternoon feeding and in 1 expt (Pig 70) the radioactive compound was given together with 10 mg carrier L histidine halfway through the meal In 1 expt. in which th effect of large doses of L-histidine was examined 10 g L-histidine monohydrochloride was given by way of a stomach tube about 2 h before feeding

Biological determination of free and conjugated Hi
Urinary free Hi was determined as previously described (Eliassen and Sjaastad 1968) Th s method involves ion exchange chromatography followed by bioassay on isolated guinea pig ileum Conjugated Hi was determined as the increase in urinary free Hi which occurred on hydrolysis under reflux with 10 N HCl for 1 1/2 h The recovery of Hi d phosphate and N acetylhistamine (10 μg/100 ml) added to the urine was the same as previously described (Eliassen in press) 74±9 and 65±14 % respectively All values are given in terms of the base and are corrected for analytical losses

Determ nat on of rad oactivity in urine and feces
Aliquots of 500 μl were transferred to scintillation vials containing 10 ml Instagel® and then counted in a Packard Tri Carb Spectrometer

The rad oactivity in feces was determined as described previously (Eliassen 1971 a) This method included combustion of 50 mg feces and trapping of the formed CO_2 in an NaOH solution

Dete minat on of radioactive Hi compounds
Immed ately after the sampl ng period was finished the analysis started The radioactive Hi m tabolites in urine were determined by isotope dilution technique (Eliassen 1969) For ^{14}C-Hi determinat ons 100—250 ml urine and 99 6 mg Hi × 2 HCl carrier were used In order to remove ^{14}C histidine from the urine about 10 mg L-histidine was added the urine was neutralized with NaOH and further one volume 0 02 M phosphate buffer pH 7 5 was added and the mixture was allowed to flow through a Dowex 1×8 column (1 2×10 cm) in acetate hydroxide form (Kremzer and Wilson 1961) The percolate was evaporated under reduced

TABLE I Quantitative analyses of radioactive and non radioactive histamine metabolites in the urine of pigs after oral administration of ^{14}C-1 Histidine

Animal	Weight of animal kg	Non radioactive compounds		Excreted radioactivity per cent of administered			Metabolites in per cent of total ^{14}C in the first 24 h urine		
		Free Hi µg	Conjug Hi µg	0–24 h	24–48 h	48–72 h	Free Hi	1 4 MeHi	1 4 MeImAA
Pig 18	70	105	24 300	34	6 3	2 7	3 4	0 5	62
Pig 25	40	45	600	12	0 6	—	0 7	0 6	47
Pig 26	55	65	2 200	23	2 9	—	1 3	0 3	36
Pig 70*	57	98	4 000	11	—	—	0 4	0 3	35

* C-L-Histidine given together with 10 mg non labelled L-histidine

pressure to a volume of about 20 ml 8 g Na SO₄ and 1 3 ml 10 N NaOH was added and Hi was extracted with n butanol The butanol layer was washed with 20 ml 0 1 NaOH containing 10 mg L-histidine and Hi was re extracted from the butanol layer with 15 ml N HCl From this point the procedure was as previously described (Eliassen 1969) For determination of the Hi metabolite ^{14}C-1 4 MeImAA the modified isotope dilution technique of Granerus was used as previously described (Eliassen 1971 a)

Materials 1 4 MeHi HCl as well as 1 4 MeImAA HCl was synthesized from Hi and L histidin respectively L-histidine (2 ring ^{14}C) spec act 44 mCi/mmol (Batch 51) and 58 3 mCi/mmol (Batch 47) was obtained from the Radiochemical Centre Amersham England Further the scintillation liquid Instagel® was purchased from Packard Instrument International S A In Zurich Switzerland

Otherwise the reagents in this study were those described previously (Eliassen 1969 and 1971 a)

Results

Urinary excretion of free and conjugated Hi

The variation in the urinary excretion of biologically determined free Hi seemed to be large but not so large as for conjugated Hi (Table I) The average amount excreted as free Hi was only about 1/3 of that found in a previous study (Eliassen 1971 b) The urinary excretion of free and conjugated histamine subsequent to oral administration of 10 mg L histidine halfway through a meal fell within the ranges observed when only ^{14}C L histidine was given between two meals The values for the control days were 99 and 3000 µg respectively

Oral administration of 10 g L-histidine gave an increase in free Hi conjugated Hi and 1 4 MeImAA but the increase was much lower than what would be expected from the experiments with radioisotopes (Table I) The values for free Hi conjugated Hi and 1 4 MeImAA when giving 10 g L histidine mono hydrochloride were 140 µg 8700 µg and 63 mg respectively The corresponding values when giving only 10 mg L histidine were 98 µg 400 µg and 44 mg

Excretion of ^{14}C activity after administration of ^{14}C L histidine

About 1/10 to 1/3 of the administered radioactivity was recovered in the first 24 h urine (Table I) The recovery was much lower than after oral administration of

^{14}C Hi (Eliassen in press) Significant amounts of radioactivity could be detected in the urine 10 days after administration The radioactivity recovered in feces of pig no 18 during the first 3 days after administration of ^{14}C L-histidine accounted for about 6 % of the given dose In the other animals the radioactivity in feces was not measured

Quantitation of ^{14}C Hi and its metabolites in the urine
Free Hi accounted for about 1 % of the urinary radioactivity while 1 4 MeHi and 1 4 MeImAA accounted for about 0 5 and 40 % respectively Giving 10 mg non labelled L-histidine together with ^{14}C L-histidine halfway through the meal did not change the metabolic pattern significantly from that observed when ^{14}C L histidine only was given between the two meals

The radioactivity in the Hi crystals when isotope dilution analyses for ^{14}C Hi were done on ^{14}C L-histidine standard instead of urine corresponded to less than 0 3 % of the radioactivity in the solution The corresponding values for 1 4 MeHi and 1 4 MeImAA were near 0 The Hi crystals made in the urine always accounted for more than 0 3 % of the urinary radioactivity

Discussion

The present experiments indicate that roughly 0 04—1 2 % of orally administrated ^{14}C L-histidine is excreted as ^{14}C Hi in the first 24 h urine A possible overestimation of ^{14}C Hi cannot be precluded One possibility for erroneously high ^{14}C Hi values is that ^{14}C-conjugated Hi which is probably present in the urine may be partly hydrolyzed to ^{14}C Hi in the isotope dilution procedure of Schayer (Granerus 1968) It is also possible that ^{14}C L-histidine is decarboxylated non-enzymatically in an acidified urine This decarboxylation is probably insignificant in the present study since the temperature of the urine was kept low during collection It is also possible that the radioactivity of the Hi crystals may partly be due to substances other than ^{14}C Hi In order to reduce the interference from ^{14}C L histidine a special procedure including ion exchange chromatography was performed in the present study However this procedure did not seem to be 100 % efficient since crystals obtained from a pure ^{14}C L histidine solution to which carrier Hi had been added accounted for 0 3 % of the radioactivity in the ^{14}C L-histidine solution

Even if the values recorded for ^{14}C Hi should be overestimated the relatively large excretion of ^{14}C 1 4 MeHi and the even higher values found for ^{14}C 1 4 MeImAA both metabolites assumed to derive from Hi only shows that orally administered ^{14}C L-histidine is to a large degree decarboxylated to ^{14}C Hi (Table I)

Using the data obtained in the present study it was calculated that 4—22 % of orally administered L histidine is converted to Hi This is far superior to what has been found in other species According to Schayer (1966) rats and guinea pigs convert about 0 2 and 0 05 % repectively of L histidine to Hi In man no radioactive Hi or known Hi metabolites were found in the urine after oral administration of L

mg ^{14}C L-histidine (Demis and Brown 1961) In 3 out of 4 expts histidine was given between meals in the present study It seemed possible that histidine in the food might be treated differently However, giving the ^{14}C L histidine together with 10 mg of carrier L-histidine halfway through the meal did not change the histamine formation significantly The much higher transformation of L histidine to Hi in pigs than that reported for other species is probably therefore real and not solely due to methodological differences Excretion of Hi and 1 4 MeImAA varies greatly (Eliassen 1971 b and Eliassen in press) The urinary increase in these 2 compounds after oral administration of 10 g L-histidine could therefore only be calculated very roughly However, there is no doubt that a smaller fraction of the larger dose was excreted as Hi and 1 4 MeImAA In man however, the opposite seemed to be true since doses of 5—10 g seemed to be necessary to obtain increased Hi formation (Irvine Duthie and Waton 1959, Oats Marsh and Sjoerdesma 1962 and Sjaastad 1966)

The quantity of L histidine ingested daily by man or pigs amounts to a few grams, but most of this derives from proteins which are slowly degraded throughout the digestion period Giving 5—10 g L histidine orally in one dose can therefore hardly be regarded to mimic a physiological situation Experiments with ^{14}C-L-histidine incorporated into proteins might give more reliable information about the decarboxylation of ingested L-histidine to Hi

With the exception of conjugated Hi in pig 18 the Hi excretion in the present experiment corresponds well with other experiments in 1972 (Eliassen in press), but is somewhat lower than found in 1971 (Eliassen 1971 b) In man variation in the diet results in large variations in the amount of 1 4 MeImAA the main urinary Hi metabolite (Granerus 1968) The great difference between the Hi contents of urine of the pigs studied in 1971 and those used in the present experiments may be due to differences in the diet as well as differences in the intestinal microflora

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From the Nobel Institute for Neurophysiology Karolinska Institutet Stockholm Sweden

Ionic Currents at Resting Potential in Nerve Fibres from Xenopus laevis Potential Clamp Experiments

By

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Abstract

ÅRHEM P, B FRANKENHAEUSER and L E MOORE *Ionic currents at resting potential in nerve fibres from Xenopus laevis Potential clamp experiments* Acta physiol scand 1973 *88* 446—454

The ionic currents in myelinated nerve fibres were measured while the membrane potential was held at its resting value and the external solution with one ionic composition was changed to a solution with a different composition Equations were derived based on the independence principle by which the net sodium potassium and chloride currents can be calculated from the change in current associated with the concentration changes The chloride currents were found to be negligibly small compared with I_{Na} and I_K The ratio I_{Na}/I_K was found to be about -5.8 which deviates significantly from -1 This deviation either shows that the independence principle does not apply to I_{Na} and/or I_K at rest or that the membrane at rest has some other current carrier in addition to Na K and Cl The slope conductance of the membrane was measured and the measured slope conductance was compared with the sum of the sodium and potassium slope conductances at resting potential calculated from the measured current changes caused by concentration changes The measured slope conductance was 2.7 times larger than the sum of the calculated ionic conductances It is concluded that resting potential of the myelinated nerve is not satisfactorily described as a potassium electrode with a sodium error

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Resting nerve has a potential difference across its membrane of about -70 mV (defined as inside potential minus potential of the external medium) The ratio of potassium concentration of the external solution to that of the axis cylinder is about 1 50 (Fenn *et al* 1934) This ratio would give a Nernst potential of about -97 mV to a potassium electrode or to a membrane specifically permeable to potassium Further it is well known that the resting potential of nerve depends on the external potassium concentration and that the potential at high potassium concentration changes almost 58 mV per tenfold concentration change as predicted by the Nernst equation It therefore seems reasonable to assume that the resting membrane is mainly permeable to potassium

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At low external potassium concentrations however the potential changes clearly less than a potassium electrode for change in $[K]_o$. This is generally taken as an indication of a small but definite permeability to sodium (Hodgkin and Katz 1949, Huxley and Stampfli 1951, Hodgkin 1964). Changes in chloride concentration are associated with very small changes in membrane potential. The chloride permeability of squid nerve is low (Caldwell and Keynes 1960, Hodgkin 1964) and may possibly be neglected in its effect on the resting membrane potential.

Changes in the internal potassium concentration also affect the resting potential (Baker, Hodgkin and Shaw 1961). Concerning these measurements on the perfused squid fibre it should be pointed out that the concentration effect on the membrane potential is small in the region of high internal potassium concentration. Such an effect may be explained as the authors point out by the dependence of the potassium permeability on the membrane potential.

Calculations have been made of the ratio between the potassium permeability and the sodium permeability of the resting membrane. These calculations were made from measurements of membrane potential at rest on the basis of the assumptions that (1) the sum of the net sodium current and the net potassium current is zero, (2) sodium and potassium move as charged particles move in free diffusion in an electric field, (3) these ions are the only current carriers. (The current carried by Cl may in addition be accounted for or may be neglected.)

The present experiments were designed so that a measurement would be obtained of the ratio between potassium, sodium and chloride currents and especially so that each measurement would be made independent of the other measurements and at a constant membrane potential. The measurements were carried out in the following manner: (a) the membrane potential of a single node in a myelinated nerve fibre was clamped at the resting value, (b) solutions with different ionic compositions were applied to the node while the change in membrane current caused by the composition change was measured, (c) a ratio for the ionic currents was calculated from these measurements.

The measurements indicated that the permeability ratio obtained did not predict the resting potential of the myelinated nerve fibre on the basis of the assumptions mentioned above.

Methods

Single large myelinated nerve fibres from the sciatic nerve of *Xenopus laevis* were used for the experiments. The membrane potential of a single node of Ranvier was controlled by a feedback amplifier system as earlier described (Dodge and Frankenhaeuser 1958, 1959). Operational amplifiers with field effect transistor inputs were used: (a) Philbrick Nexus 10110[illegible] as input followers and (b) Philbrick Nexus (100[illegible]01 as the two feed back amplifiers. This modification allowed an increased signal to noise ratio compared to that obtained with the electronic valve amplifiers. This was essential for the measurements of the small changes in membrane current associated with the solution changes made when membrane potential was clamped to its resting value.

Regenerative activity. An all or none current response was seen in some long lasting experiments. The amplitude time course of this response was the same as that of an action potential. It had a sharp threshold and an appreciable latency at pulses close to threshold. The response

was associated with a negligible change in input potential at the potential monitoring amplifiers It appeared more often at higher temperatures and after solution changes This type of response was not seen in earlier experiments and it was evident that this behaviour was abnormal

A high impedance in series with the membrane could obviously allow the membrane to fire an all or none response that would not be prevented by a high gain in the potential controlling feed back amplifiers Such a high impedance might as an example be caused by some petroleum jelly droplets sticking to the outside of the node Tests showed that the artifact could be produced by a slight touch of the node by some petroleum jelly or by letting the node touch the solution surface which was covered by some petroleum jelly It was not possible to reveal why this artifact appeared in these experiments but not during the earlier years A slight difference in the composition of the petroleum jelly used now and earlier might be the reason for this unexpected behaviour or it may be caused by a slight drifference in the shape of the recording cell causing petroleum jelly droplets to reach the fibre in an unexpected manner Since the artifact clearly causes errors in the measurements when it appears the experiments were therefore interrupted and measurements were discarded when the artifact appeared

Solutions Solutions with a number of different sodium potassium and chloride concentrations were used (see Table 1) Every solution contained 2 mM $CaCl_2$ and either 2 5 mM $NaHCO_3$ or 5 mM tris (hydroxymethyl) aminomethane as buffer The nodal current was buffer independent at corresponding pH and otherwise unchanged composition of solution

Some experiments were made with the metabolic inhibitors 2 4-dinitrophenol (DNP) or strophanthin G (ouabain) (both substances from Sigma Chemical Company) added in various amounts to the Ringer solution Solutions with DNP were adjusted with tris base to pH = 7 0

Nomenclature and symbols Membrane potential (E) is given as axis cylinder potential minus outside potential Outward membrane current is consequently positive

I I_{Na} I_K I_{Cl}	ionic current as defined by subscripts
$[Na]_o$ $[K]_o$ $[Cl]_o$	ionic concentration of external solution
$[Na]_i$ $[K]_i$ $[Cl]_i$	ionic concentration of axis cylinder
ΔI_{Na} ΔI_K ΔI_{Cl}	change in ionic current
$\Delta[Na]_o$ $\Delta[K]_o$ $\Delta[Cl]_o$	change in concentration of external solution
G G_{Na} G_K	membrane slope conductance as defined by subscripts $G = \Delta I/\Delta E$
P_{Na} P_K	permeability as defined by the constant field equation
R	gas constant
T	absolute temperature
F	Faradays constant
J	ionic strength
f	activity coefficient
z	valency

Principle of analysis

The aim of the present investigation was to obtain independent determinations of each of the ionic currents (I_{Na} I_K and I_{Cl}) at resting membrane potential and to check whether the sum of the so determined currents equalled zero or not

The experiments were carried out so that the membrane potential was held clamped at its resting value or in some cases at other fixed values while the external solution with one composition was changed to a solution with a different composition The change in membrane current (ΔI) associated with the solution change was measured

The change in current caused by a certain change in concentration cannot be a direct measure of the current carried by a single type of ion However some general assumptions allow a calculation of the net current from such measurements We assume (a) that the chance that any individual ion will cross the membrane in a specified interval of time is independent of the other ions which are present (the independence principle Hodgkin and Huxley 1952a) and (b) that the membrane permeability (P_{Na} P_K and P_{Cl}) is unaffected by the concentration changes.

On the basis of the independence principle (see Hodgkin and Huxley 1952a) the sodium current at the concentration $[Na]$ can be calculated from measurements of the change in I_{Na} due to the change in sodium concentration

$$I_{Na} = \frac{\Delta I_{Na}}{\Delta[Na]_o}\left[[Na]_o - [Na]_i \exp\{EF/RT\}\right] \tag{1}$$

I_K and I_{Cl} can similarly be calculated from measured current changes caused by concentration changes of $[K]_o$ respectively $[Cl]_o$ and correspondingly modified equations

TABLE I Composition of solutions

Solution	Na+ m equiv/l	K+	Cl-	CH SO4	Sucrose mM
1	114 5	2 5	118 5	—	—
2	114 5	2 5	118 5	—	240
3	114 5	2 5	6 5	112 0	—
4	2 5	2 5	6 5	—	240
5	65 0	2 5	69 0	—	—
6	105 0	2 5	109 0	—	—
7	125 0	2 5	129 0	—	—
8	177 0	2 5	181 0	—	—
9	177 0	2 5	118 5	62 5	—
10	234 5	2 5	118 5	120 0	—
11	114 5	10 0	126 0	—	—
12	114 5	20 0	136 0	—	—
13	114 5	120 0	236 0	—	—
14	114 5	190 0	306 0	—	—
15	114 5	65 0	118 5	62 5	—
16	114 5	122 5	118 5	120 0	—
17	114 5	190 0	118 5	187 5	—
18	5 0	120 0	126 5	—	—
19	65 0	65 0	131 5	—	—

The equation is written for simplicity with concentrations Calculations were also made with activity which is more correct. The activity coefficient (f) was then estimated by equation (2) which is held to be a reasonable approximation for strong electrolytes and ionic strengths below 0 3 (Edsall and Wyman 1958) Fig 1 is a solution of equation 2

$$\log f = z_+ z_- \, 0\,5 \sqrt{\omega} \,/(1 + \sqrt{\omega}) \qquad (2)$$

Experimental determinations were also made of the slope conductance of the resting membrane $G = \Delta I / \Delta E$ from the change in membrane current associated with a small change in membrane potential If it is asumesd that sodium, potassium and chloride are the only carriers of current through the membrane then it follows that

$$G = G_{Na} + G_K + G_{Cl} \qquad (3)$$

where $G_{Na} = \Delta I_{Na} / \Delta E$ $G_K = \Delta I_K / \Delta E$ and $G_{Cl} = \Delta I_{Cl} / \Delta E$ The individual ionic slope conductances could not be obtained without further assumptions The constant field assumption (see Goldman 1943 and Hodgkin and Katz 1949) allows calculation of these ionic permeabilities from the measurements of ΔI associated with concentration changes

$$P_{Na} = \frac{\Delta I_{Na}}{\Delta [Na]_o} \frac{RT\ (1-\exp\{EF/RT\})}{EF^2} \qquad (4)$$

P_K and P_C were determined correspondingly The sodium slope conductance ($G_{Na} = \Delta I_{Na} / \Delta E$) could then be calculated because $\Delta I_{Na} = I_{Na}' - I_{Na}$ where I_{Na} is the sodium current at the potential E and I_{Na}' the sodium current at $E + \Delta E$ The currents can be calculated from the permeability by the constant field equation Combining these steps we obtain

$$G_{Na} = \frac{P_{Na}}{\Delta E} \frac{F^2}{RT} \left[\frac{(E+\Delta E)[Na]_o - [Na]_i \exp\{(E+\Delta E)\ F/RT\}}{1 - \exp\{(E+\Delta E)\ F/RT\}} - \frac{E([Na]_o - [Na]_i \exp\{EF/RT\})}{1 - \exp\{EF/RT\}} \right] \qquad (5)$$

G_K and G_{Cl} were determined correspondingly

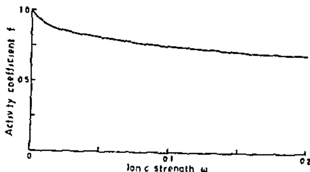


Fig 1 Activity coefficient (f) plotted against ionic strength (ω) as solution of equation 2

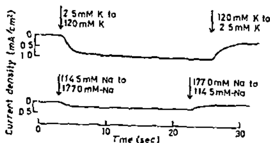


Fig 2 Membrane currents associated with changes in external medium Major changes as indicated by arrows Solutions used 1 13 8 Temp °2 C

Results

The isolated nerve fibre was mounted in the recording cell the electrodes were connected to the solution pools in the cell the feed back amplifiers were balanced and the membrane potential was clamped close to its resting value (*e g* Dodge and Frankenhaeuser 1958) Changes in the nodal membrane current were recorded while solutions with various compositions (see Table I) were applied to the node (N_o) under investigation

Experiments were carried out where the ordinary Ringer solution was replaced by a solution with increased osmolarity (solution 2) The osmolarity was increased by sucrose while the ionic composition of the solution was the same as in the reference solution No appreciable current change (< 0 05 mA/cm) occurred in association to this change in osmolarity of the external solution This finding has some bearing on the experiments to be described because it shows that solutions with various osmolarities may be used without any direct fast osmotic effects on the membrane currents Test solutions were applied for a short time only (not more than about half a minute) in order to avoid slow effects of changes in tonicity of the test solutions and consequent changes of the ionic concentrations of the axis cylinder

The next procedure was to measure the membrane current associated with changes between solutions of different ionic compositions An increase in $[Na]_o$ as well as in $[K]_o$ caused an inward current (Fig 2) The current transient lasted between half a second and about two seconds and clearly depended on the rate of solution change No further steps were taken to investigate this rate of current

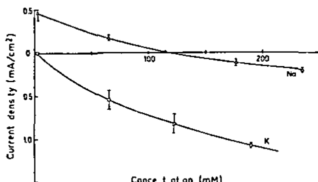


Fig 3 Measured changes in membrane current associated with changes between solutions of different compositions Ordinates given as current in test solution minus current in ordinary Ringer solution (solution 1) Mean and S.D N = 5 Abscissae give concentration of [Na]o for upper curve and of [K]o for lower curve Temp 22 C

change Since the dead space in the cell was relatively large it seems likely that the rate was limited by the rate of the solution change A very small slow change in current was sometimes noted in addition to the rapid change The resolution of the technique was not sufficient to investigate this slow creep in further detail The [Cl] was not held constant in these solutions and therefore it was possible that some of the current change was caused by the change in $[Cl]_o$ In order to check this possibility runs were made with a solution where nearly all the chloride was replaced by CH_3SO_4 (solution 3) This change in composition caused a minor current change only ($<$ 0 05 mA/cm²) It was therefore concluded that a negligible fraction only of the membrane current was carried by Cl An alternative explanation to the finding is that the chloride permeability and the methylsulphate permeability would be equal This however seems unlikely

Measurements were carried out of the currents associated with concentration changes of sodium and potassium A wide range of concentrations was used Fig 3 is an example of such current measurements on five axons It shows that $\Delta I/\Delta[Na]$ and $\Delta I/\Delta[K]$ are both negative over the whole range of concentrations This was expected because an increase of the positive ions on the outside of the membrane should increase the inward flux of these ions and the potential-current nomenclature used defines this current change as negative Further it is evident from the figure that $\Delta I/\Delta[Na]_o$ and $\Delta I/\Delta[K]$ were larger at low concentrations than at high concentrations When a similar plot was made with activities (using equation 2) instead of concentrations the relation was somewhat more linear but a definite curvature was still regularly noted This experimental finding was neither expected nor was it predicted by the independence principle (compare equation 1)

Measurements of this kind were made on several nerve fibres The size of the net sodium current and the net potassium current was then calculated (equation 1) from these measurements of $\Delta I_{Na}/\Delta[Na]$ and $\Delta I_K/\Delta[K]$ on each individual fibre at the concentrations in the reference Ringer solution (*i e* 112 mM NaCl and 2 5 mM KCl) The derivates required for equation 1 were obtained from the smooth curves drawn through a graph of the experimental points The ratio I_{Na}/I_K for each individual fibre was then calculated A mean value of $-5\,9 \pm 2\,1$

TABLE II Membrane conductances G_N and G_K obtained by equation (5) $G = G_{Na} + G_K$ G determined as $\Delta I / \Delta E$ All conductances are given as m S/cm Axis cylinder concentrations taken as 15 m-equiv Na /l and 120 m equiv K /l

Fibre	G_N	G_K	G	G	G /G
1	5 5	6 0	11 5	35	3 0
2	6 3	4 0	10 3	33	3 2
3	5 5	3 5	9 0	20	2 2
4	7 4	5 4	12 8	27	2 1
5	2 9	6 2	9 1	30	3 3
6	5 1	4 6	9 7	22	2 3
				Mean	2 7

(mean ± S D) was obtained from measurements on 15 fibres The numerical value of this ratio is significantly larger than one which is the value expected on the basis of the assumptions that (a) the sodium current and the potassium current in the resting fibre follow the independence principle and (b) all other currents are so small that they can be neglected

The resting potential of nerve is generally in text books etc held to be caused by a large potassium permeability and a relatively small sodium permeability The findings just described are in conflict with this commonly accepted idea Because of this it seemed important to do further experiments in order to avoid possible mistakes In the experiments just described the fibre was cut off at the internodes neighbouring the node (N_0) under investigation This was made in order to obtain a constant impedance in the current recording path and a low impedance from the axis cylinder at node N_0 to the input of the first feed back amplifier This procedure of cutting the fibre might however change the inside concentrations Even an assumed increase of $[Na]_i$ to 50 mM would decrease the calculated sodium current only about 2 % Correspondingly quite non realistic inside potassium concentrations (500 mM) would be required for an agreement between the experimental findings and $I_{Na} + I_K$ beeing zero on the basis of the above assumptions

In spite of this prediction we decided to do a check with measurements on fibres with intact excitable nodes on both sides of that under investigation The calculated ratio I_{Na}/I_K in these experiments was $-5\,4 \pm 1\,5$ (mean and S D n = 4) which result is in good agreement with the results on the cut fibres

Furthermore experiments were made to determine the potential dependence of the ratio I_N/I_K These showed that the ratio was almost independent of potential in the range -60 to -90 mV

Measurements were also made of the membrane slope conductance G defined as $G = \Delta I/\Delta E$ The unit for G reciprocal ohm is called Siemens and abbreviated S according to the SI system The change in net membrane current associated with a potential step was measured for the determination of membrane slope conductance This conductance should be the sum of the ionic slope conductances (equation 3) The ionic permeabilities were determined from the measurements of current changes associated with concentration changes by equation 4 and the ionic slope

conductances were calculated by equation 5 We thus obtain two determinations of membrane conductance one from the current change associated with a small potential step and the other from measurements of current changes associated with concentration changes Table II shows a clear discrepancy between the two conductances

One obvious explanation to the discrepancy between these independent measurements would be that membrane current would be carried by an electrogenic ion pump In order to obtain at least some evidence on this point experiments were made in which the metabolic inhibitor 2 4 dinitrophenol (DNP) or ouabain was applied to the clamped node Neither 20 10^{-3} % DNP nor 8 10^{-3} % ouabain caused any detectable change in membrane current within 15 min

Discussion

One of the main pieces of evidence for regarding the resting potential as at least partly due to the potassium concentration cell is that at high external potassium concentrations the membrane behaves like a potassium electrode (Hodgkin 1964) It is well known that the potassium permeability of the nerve membrane depends on the membrane potential (*e g* Hodgkin and Huxley 1952 b Frankenhaeuser 1963) The behaviour like a potassium electrode might therefore, apply to a fibre with low membrane potential only An indication of this kind was obtained by Stampfli (1959) when he applied currents to myelinated nerve fibres in various external solutions The present investigation was carried out in order to obtain independent measurements of I_{Na} and I_K in the resting fibre at resting potential and thus to get some clue about the permeability conditions of the resting fibre

The currents at resting potential associated with concentration changes are small and so are the currents caused by small potential steps The signal to noise ratio earlier obtained was too small for reliable measurements The resolution was clearly increased with the present clamp instrumentation

The measurements showed that $\Delta I/\Delta[Cl]$ was negligibly small compared to $\Delta I/\Delta[Na]$ and $\Delta I/\Delta[K]$ It is therefore concluded that Cl and P_{Cl} do not play any significant role for maintaining the resting potential An equation was derived to calculate I_{Na} and I_K from the measurements of the current changes associated with concentration changes (equation 1) This equation was derived from the independence principle (Hodgkin and Huxley 1952 a) for the condition of these measurements without any further assumptions It therefore holds for all the conditions to which the independence principle applies (see Frankenhaeuser 1960) The finding that the ratio I_{Na}/I_K deviates quite regularly from -1 either shows (1) that the independence principle does not apply to the sodium and/or potassium currents at rest or (2) that the nodal membrane at rest has some other current carrier in addition to Na K and Cl The present experiments do not distinguish between these alternatives A possible unknown current carrier might be an electrogenic ion pump Some of the well known metabolic inhibitors were therefore tried These inhibitors did not cause a change in membrane current a finding which

is consistent with the finding of Schoepfle and Bloom (1959) that resting potential is unaffected by cyanide and DNP The finding does however, not exclude an electrogenic pump which is resistant to these inhibitors

An electrogenic pump might be unaffected by the electric driving force *i e* behaving as a constant current device In that case the membrane slope conductance would be unaffected by the pump It seemed therefore worth while to obtain an expression for the sodium and the potassium slope conductance (equation 5) and compare the sum of these with the membrane slope conductance This comparison showed that the measured membrane conductance was larger than the sum of the two calculated ionic conductances (Table II) The conclusion from this is either (a) that the constant field equation does not apply to one or both of the ionic currents or (b) that the membrane has some conductance in addition to G_{Na}, G_K and G_{Cl} It was impossible to decide between these two alternatives It may in this connection be pointed out that the leak conductance, g_l is linear with potential the mechanism for this is not known

The final conclusion of the present paper is that the resting potential of the myelinated nerve is not satisfactorily described as a potassium electrode with a sodium error

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Histochemical Demonstration of Carbonic Anhydrase Activity in the Human Kidney

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Abstract

LONNERHOLM G *Histochemical demonstration of carbonic anhydrase activity in the human kidney* Acta physiol scand 1973 *88* 455—468

The distribution of carbonic anhydrase activity along the tubules of fresh human donor kidneys prepared for transplantation was studied by the histochemical method of Hansson Enzyme activity was demonstrated in the cells of the convoluted part of the proximal tubule including the brush border region The straight part of the proximal tubule contained little or no enzyme indicating a functional differentiation of the two parts of the proximal tubule The staining of the thin limb was different in fixed and unfixed tissue The straight and convoluted parts of the distal tubule however showed distinct enzyme activity In the major part of the collecting duct a mosaic of cells was found some cells with high and other cells with no or low enzyme activity In the most distal part near the papilla, however no enzyme activity was found The present study shows that carbonic anhydrase is present in those parts of the kidney tubules where there is a postulated role for it The distribution of the enzyme in the human kidney is similar to that seen in the rat and dog kidney which suggests a common pattern of urinary acidification in these species

The distribution of carbonic anhydrase (carbonate hydrolase EC 4 2 1 1) in the human kidney has hitherto been studied by determining the enzyme activity by biochemical techniques in dissected parts of the kidney (Wistrand and Wahlstrand personal communication Mattenheimer *et al* 1970) There is however no detailed picture of the enzyme activity in the different part of the human kidney tubules and in single cells Information of this kind is necessary in order to understand the acidifying processes along the tubules as discussed by Pitts (1968) and Rector (1971) and also for the discussion of the renal effects of carbonic anhydrase inhibitors including the thiazide diuretics In the present study a recently introduced histochemical method has been used Some results have been published in a preliminary form (Lonnerholm 1972)

Methods

8 human kidneys were examined 7 were donor kidneys (no 1—7, Table I) prepared for transplantation There was no history of renal disease Although data are not complete in no case is it known that thiazide diuretics or other drugs of importance had been administered to the donors Immediately after removal the kidneys were perfused with 400—1000 ml of a cold Ringer-dextran type of solution (Perfadex® Pharmacia, Uppsala Sweden) containing 10 million IU benzylpenicillin 50 ml 2 % lidocaine (Xylocain® Astra, Sodertalje Sweden) and 5000 IU heparin per 1000 ml solution This was followed by perfusion with 400—1000 ml of a 10 % invertose solution (Inverdex® Pharmacia) to which sodium bicarbonate had been added The perfusion pressure was 150 mm Hg Transplantation of the kidneys was not performed due to presence of multiple renal vessels incomplete perfusion warm ischemic period exceeding 50 min or difficulties to find a suitable recipient The kidneys were stored at 4 C and tissue preparation started within 24 h of perfusion

One kidney with a small tumour in the renal pelvis (no 8 Table I) was removed by nephrectomy and perfused with a cold Ringer type of solution (Salidex® Pharmacia) whereupon the tissue preparation started immediately

Preparation of tissue

Unfixed tissue was obtained from five kidneys (no 1—3 6 7) Small tissue blocks were dissected from different regions of the kidney and then immediately frozen in isopentane cooled with liquid nitrogen. Badly perfused regions were avoided Sections were cut at −20 C and handled in one of two ways

1) 4 μm sections were thawed on a TH WP Millipore filter 25 μm thick pore size 0 45 μm (Millipore Filter Corporation Bedford Mass USA) and then immediately stained

2) 15 μm sections were freeze dried for 1 h at 0 03 mm Hg and −50 C and then stained These sections could be stored for some days before staining

Fixed tissue was obtained from all kidneys except no 3 Dissected tissue blocks (maximum thickness 4 mm) were fixed by immersion in 2 5 % glutaraldehyde in 0 1 M phosphate buffer at pH 7 4 The glutaraldehyde was prepared from a 50 % stock solution by a one stage vacuum distillation (Andersson 1967) The purity of the distillate was checked by ultraviolet spectrophotometry The tissue specimens were incubated in the fixative for 16 h at 4 C and then washed in 0 2 M sucrose in 0 05 M phosphate buffer at pH 7 4 for 4 h at 4 C to remove excess fixative (Kidneys no 4 5 8 were perfused with fixative through the renal artery with or without subsequent immersion of dissected tissue blocks in fixative This modification did not significantly improve the preservation of cellular structure) The blocks were thereafter frozen in isopentane cooled with liquid nitrogen and sectioned at −20 C The sections were handled in one of two ways

1) 4 μm sections were thawed on Millipore filters and stained

2) 8 μm sections were collected in a Petri dish containing cold buffered sucrose (see above) and were then transferred to the incubation medium within 3 min

Staining procedure

The sections were stained for carbonic anhydrase activity according to the method of Hansson (1967 1968) The sections were floated on the surface of the freshly prepared incubation medium in a 9 5 cm Petri dish The medium contained $CoSO_4$ 1 75 × 10^{-3} M H_2SO_4 53 × 10^{-3} M $NaHCO_3$ 0 157 M and KH_2PO_4 11 7 × 10^{-3} M A compound containing cobalt and phosphorous precipitates at carbonic anhydrase sites and is converted to CoS Thus a black precipitate is formed where enzyme is present The volume of the incubation medium was 57 ml and always included 10 ml 1/15 M KH_2PO_4 (= 11 7 × 10^{-3} M) to ensure maximal sensitivity and short incubation times 1—9 min for free floating sections and 3—12 min for sections on Millipore filters

The sections on Millipore filters were allowed to equilibrate with the incubation medium for 10 min under a carbon dioxide atmosphere The use of Millipore filters instead of free floating sections made it necessary to use slightly longer incubation times but the thin sections on the Millipore filters permitted better resolution

When free floating sections were used the unfixed freeze-dried sections were somewhat more intensely stained than the fixed ones but the staining was less distinct and the sections tended to disintegrate To prevent the free floating sections from disintegrating in the slightly effervescent incubation medium the non ionic detergent Tween 20 was added to a final concentration of 1/100 000 (v/v)

Some sections were counterstained with haematoxylin and eosin In some experiments every other section was stained for carbonic anhydrase activity and every other for mitochondria with anilin acid fuchsin methyl green according to Lillie (1954) Pairs of sections 4 μm thick were matched in this way

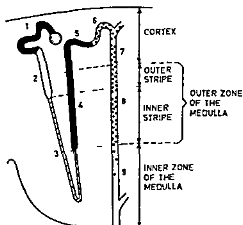


Fig 1 1) Proximal tubule convoluted part 2) proximal tubule straight part 3) thin limb 4) distal tubule straight part 5) distal tubule convoluted part 6) collecting duct initial segment 7) collecting duct medullary ray segment 8) collecting duct outer medullary segment 9) collecting duct inner medullary segment Solid black represents positive staining for carbonic anhydrase activity Dotted areas are regions with a mosaic of stained and unstained cells Striped areas are regions where conflicting results were obtained (see text)

Control experiments
1) Sections from tissues where the enzymatic activity had been destroyed by boiling were unreactive
2) When the inhibitors acetazolamide 10^{-5} M (Diamox® American Cyanamid Company Pearl River N Y USA) or ethoxzolamide 10^{-6} M (Cardrase® The Upjohn Company Kalamazoo Mich USA) were included in the incubation medium no staining occurred in either fixed or unfixed sections With acetazolamide 10^{-6} M the staining was much delayed and weakened but not completely inhibited
Further evidence in favour of the specificity of the method will be presented in a separate study (Lonnerholm in preparation)

Microphotography
Microphotographs were taken on Kodak Panatomic X film using a Zeiss standard microscope GFL with a Zeiss photo attachment

NOMENCLATURE
The different parts of the kidney tubules (see Fig 1) were named according to Trump and Bulger (1968) (In a paper describing the distribution of carbonic anhydrase in the rat kidney (Lonnerholm 1971) a somewhat different nomenclature was used) The figures in brackets refer to Fig 1 The convoluted (1) and straight part (2) of the proximal tubule the thin limb (= the thin segment of Henle s loop) (3) the straight (4) and convoluted part (5) of the distal tubule (the straight part is also known as the ascending thick segment of Henle s loop) and the collecting duct The collecting duct is divided into four segments initial (6) medullary ray (7) outer medullary (8) and inner medullary segment (9) The initial segment (also named connecting portion or arched collecting tubule) begins at the end of the distal tubule in the cortical labyrinth and passes to the medullary ray
According to Peter (1909) the kidney is divided into cortex outer zone of the medulla composed of the outer stripe and the inner stripe and inner zone of the medulla The cortical substance is made up of the cortical labyrinth and the medullary rays
The cortical labyrinth consists of renal corpuscles the convoluted part of the proximal tubule the convoluted part of the distal tubule and the initial segment of the collecting duct
The medullary rays which penetrate into the cortex consist of the straight part of the proximal tubule the straight part of the distal tubule and collecting ducts

Results

Histological appearance of the kidney tissue
When human kidney tissue is fixed by immersion of small cubes of tissue the lumina of the proximal tubules appear collapsed or obliterated by cellular debris (Tisher

Table I Characteristics of the kidneys

Kidney no	Age of donor (years)	Sex	Length of warm ischemic period (min)
1	47	male	45
2	25	male*	50
3	44	male**	50
4	44	male**	60
5	34	female	50
6	12	male	20
7	8	male	30
8	75	male	45

* Hypotension and anuria during the 24 h before death
** No 3 and 4 obtained from the same donor

et al 1966 Ericsson *et al* 1965 1967) However, in the present study, the proximal tubules generally showed patent lumina both in fixed tissue, Fig 2 P and unfixed tissue, Fig 3 P Evidently the initial perfusion with Perfadex® solution caused the tubules to remain open It is now generally agreed that the proximal tubules in functioning kidneys have patent lumina (see Maunsbach 1966) Apical cell protrusions were commonly seen in the proximal tubules Fig 2 P arrows They may be regarded as artifacts since they are not seen in rat kidneys fixed under optimal conditions (Maunsbach 1966 Tisher *et al* 1966) The other parts of the kidney tubules generally showed patent lumina and distinct luminal cell borders apical protrusions were only infrequently seen Large extracellular spaces were not seen in any part of the kidney

In some kidneys specially no 1 7 8 cellular structure was better preserved than in others This could be due to the clinical state of the donor during the period just before the removal of the kidney to a shorter warm ischemic period or to better initial perfusion

Cellular structure was for the most part better preserved in fixed than in unfixed tissue but many cells exhibited a somewhat washed out appearance even after glutaraldehyde fixation

Staining for carbonic anhydrase activity

Fig 1 summarizes the results of the histochemical staining for carbonic anhydrase activity in the different parts of the kidney tubules

The renal corpuscle

Bowman s capsule was always unstained

The *glomeruli* were always unstained in unfixed sections but in fixed tissue from kidneys no 1, 2 5 7 slight to moderate staining was observed The cause of the staining is not clear at present Mattenheimer *et al* (1970) detected some enzyme activity in dissected glomeruli This finding might be explained by contaminating erythrocytes since nonperfused kidneys were used Available data are not conclusive



Fig 2 Kidney no 1 Staining for carbonic anhydrase no counterstain Cortical labyrinth D = distal tubule convoluted part P = proximal tubule convoluted part Arrows point at apical protrusions Fixed 8 µm thick section Incubation time 3 min

Fig 3 Kidney no 1 Staining for carbonic anhydrase no counterstain Cortical labyrinth D = distal tubule convoluted part *or* initial segment of collecting duct P = proximal tubule convoluted part Unfixed 15 µm thick section. Incubation time 3 min

Fig 4 Kidney no 8 Staining for carbonic anhydrase no counterstain Proximal tubule convoluted part. Arrows point at positively stained brush border region Fixed 8 µm thick section Incubation time 6 min

as to the presence of enzyme in the glomeruli In rat kidneys fixed under optimal conditions the glomeruli are always unstained (Lonnerholm 1971)

The proximal tubule

The main characteristics of the proximal tubule were the large diameter sparse nuclei located basally and tall columnar cells It was also usually recognized in fixed sections by its brush border In unfixed sections however the brush border was poorly preserved

The cells of the *convoluted part* were distinctly stained Fig 2 P 4 P 5 P (fixed) and Fig 3 P (unfixed) In some of the fixed sections the brush border was well preserved and showed marked black deposits Fig 4 arrows In unfixed sections only traces of the brush border were observed and its carbonic anhydrase activity could not be evaluated Fig 3 The staining was evenly distributed in the cytoplasm Beneath the brush border in the cell apex however there was an unstained region

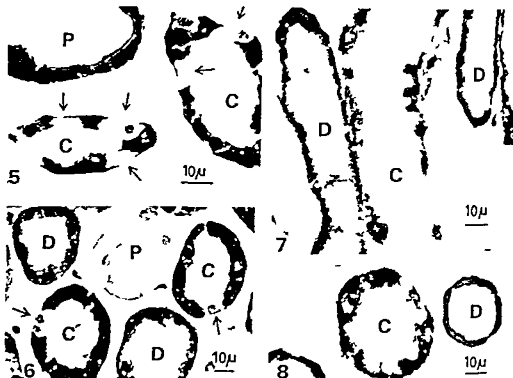


Fig 5 Kidney no 8 Staining for carbonic anhydrase no counterstain Cortical labyrinth C = collecting duct initial segment. P = proximal tubule convoluted part Arrows point at unstained or weakly stained cells Fixed 8 μm thick section Incubation time 6 min

Fig 6 Kidney no 4 Staining for carbonic anhydrase counterstaining with haematoxylin and eosin Medullary ray C = collecting duct D = distal tubule *straight part* P = proximal tubule straight part Arrows point at unstained cells Fixed 8 μm thick section Incubation time 6 min

Fig 7 Kidney no 1 Staining for carbonic anhydrase no counterstain Medullary ray C = collecting duct D = distal tubule traight part The empty space at the lower right is the unstained straight part of proximal tubules Unfixed 4 μm thick section Incubation time 6 min

Fig 8 Kidney no 1 Staining for carbonic anhydrase no counterstain Inner stripe of the outer medullary zone C = collecting duct D = distal tubule straight part Unfixed 15 μm thick section Incubation time 6 min

Fig 4 5 probably corresponding to the apical cell region known to contain many vesicles and large vacuoles (Tisher *et al* 1966)

In the *straight part* the cells were always completely unstained in unfixed sections Fig 7 empty space In fixed sections they were also generally unstained Fig 6 P but in kidneys no 1 and 5 a weak staining reaction was seen This staining occurred only after long incubation times and may represent an artifact But it is also possible that this part of the kidney tubules is low in enzyme and therefore close to the detec tion threshold

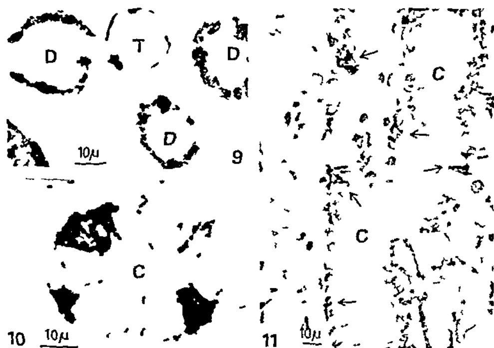


Fig 9 Kidney no 1 Staining for carbonic anhydrase no counterstain Inner stripe of the outer medullary zone D = distal tubule straight part T = thin limb Fixed 4 μm thick section Incubation time 9 min

Fig 10 Kidney no 1 Staining for carbonic anhydrase no counterstain Inner medullary zone (upper part) C = collecting duct Fixed 4 μm thick section Incubation time 3 min

Fig 11 Kidney no 1 Staining for carbonic anhydrase counterstaining with haematoxylin and eosin Inner medullary zone (middle part) C = collecting duct The confluence of two collecting ducts is shown Arrows point at the few positively stained cells Unfixed 4 μm thick section Incubation time 9 min.

The thin limb

The thin limb when transected demonstrates a low cuboidal epithelium and often 2–3 nuclei Fig 9 T Thin limbs might be mistaken for capillaries but the transected capillary shows only single nuclei and endothelial cells with very thin cytoplasm (Peter 1909) Erythrocytes inside vessels are helpful for the identification but occurred sparingly Sometimes the transition of a proximal tubule into a thin limb was observed permitting a certain identification of the thin limb

The thin limb exhibited different types of staining depending on the preparation of the tissue In fixed sections black deposits were found both in the thin cytoplasm and in the nuclei bulging into the lumen Fig 9 T Stained thin limbs were observed in the inner stripe and the inner zone of the medulla but it was not possible to distinguish between descending and ascending limbs In unfixed sections the thin limbs were always unstained It is not clear at present if this discrepancy was caused by fixation artifacts or by loss of enzyme from the unfixed sections by diffusion In



Fig 12 Kidney no 4 Staining for carbonic anhydrase counterstaining with haematoxylin and eosin Inner medullary zone (papillary region) C = collecting duct Fixed 8 μm thick section. Incubation time 6 min

rat kidneys fixed under optimal conditions the same discrepancy was observed (Lonnerholm 1971)

The distal tubule

The *straight part* and the collecting duct are located side by side The distal tubular cells are filled with many mitochondria whereas most cells in the collecting duct have a clear cytoplasm containing *few mitochondria* *The staining of mitochondria* with aniline acid fuchsin methyl green discriminated nicely between these two parts of the kidney tubules The cells in the straight part of the distal tubules showed enzyme activity most intensely in unfixed sections Fig 6 D 9 D (fixed) and 7 D 8 D (unfixed) The intensity of the staining did not change noticeably along the course of this part of the distal tubule

The *macula densa region* could not be identified and therefore very probably does not differ from the adjacent regions

The *convoluted part* could not be identified with certainty However in some tubules of the cortical labyrinth every cell was intensely stained all over the cytoplasm like the straight part of the distal tubule These tubules are thought to be identical with the convoluted part of the distal tubule Fig 2 D (fixed) When

studied with the electron microscope the cells in the straight and convoluted parts of the distal tubules appear much alike in the human kidney (Tisher *et al* 1968)

The collecting duct

Some tubules of the cortical labyrinth contained one kind of cells which was heavily stained and another kind of unstained cells The stained cells seemed to be more numerous than the unstained ones Intermediate forms showing weak staining or staining only in the basal part of the cell, were also seen The different kinds of cells were clearly seen in fixed sections Fig 5 C but they were sometimes difficult to see in unfixed sections Fig 3 D These cells are thought to be located in the *initial segment of the collecting duct* this matter is further discussed later (see Discussion)

The *medullary ray segment* is typically located in the periphery of the rays (see Peter 1927) Heavily stained cells completely unstained cells and intermediate forms were found here Fig 6 C (fixed) and Fig 7 C (unfixed) The stained cells were more numerous than the unstained ones

The *outer medullary segment* was easily identified by the presence of high columnar cells and wide lumina The cells showed the same staining as the cells in the medullary ray segment Fig 8 C (unfixed) with more stained cells than unstained

Many cells in the uppermost part of the *inner medullary segment of the collecting duct* were intensely stained all over the cytoplasm, Fig 10 C but more distally the stained cells were less frequent Fig 11 C The papillary half or third of the inner medullary segment was completely unstained Fig 12 C

The *renal interstitium* was always unstained This was most clearly seen in the medulla Fig 8—12 where the interstitium is abundant

The *nuclei* were unstained in unfixed sections In fixed sections however the nuclei were stained in those cells where the cytoplasm showed carbonic anhydrase activity but not in those where the cytoplasm was unstained Fig 5 6 10 This staining of the nuclei could be completely inhibited by acetazolamide or ethoxzolamide but its specificity has been questioned (Hansson 1967 1968)

Discussion

Comparison with results obtained by biochemical methods

Mattenheimer *et al* (1970) measured carbonic anhydrase activity in the glomeruli proximal and distal convolutions medullary rays outer and inner zone of the medulla and papilla dissected from frozen dried sections of the human kidney Wistrand and Wahlstrand (personal communication) measured enzyme activity in homogenized tissues taken from various regions of the same kidneys as used in the present study Generally the findings in the present work agree well with data from these two studies Further some biochemical data can be better understood on the basis of the present study thanks to the more detailed picture obtained by the histochemical method

Thus the *convoluted part of the proximal tubule* was intensely stained histochemically and even more so the *convoluted part of the distal tubule* This corresponds to the high enzyme activities demonstrated in the cells of the proximal and distal convolutions by Mattenheimer *et al* (1970) and to the high enzyme activity in the cortical portion shown by Wistrand and Wahlstrand

The *medullary rays* are made up to a considerable extent of the straight part of the proximal tubule and this part of the nephron was unstained or weakly stained histochemically This should explain the low enzyme activity of the medullary rays as found by Mattenheimer *et al* (1970)

The straight part of the distal tubule and collecting ducts form the bulk of the *outer medullary zone* These two parts of the kidney tubules were distinctly stained histochemically which correlates well with the high activity of enzyme demonstrated in this zone (Mattenheimer *et al* 1970)

The *inner medullary zone* contains only thin limbs and collecting ducts In the latter the histochemically stained cells were less frequently seen towards the papilla and finally disappeared completely Thin limbs might contribute some enzyme to the inner medullary zone even close to the papilla Wistrand and Wahlstrand as well as Mattenheimer *et al* (1970) also found decreasing amounts of the enzyme toward the papilla using biochemical techniques

The physiological role of carbonic anhydrase in the kidney

How does the distribution of the enzyme along the kidney tubules relate to the physiological needs *i e* to the acidifying processes as we presently know them? The excretion of hydrogen ions in the different parts of the human kidney cannot be studied by direct methods but in the rat micropuncture studies have indicated where along the kidney tubules urinary acidification takes place (Gottschalk *et al* 1960 Rector *et al* 1965 Malnic *et al* 1972) Such studies have also been done although less extensively in the dog showing a similar pattern of acidification as in the rat (Bernstein and Clapp 1968

The distribution of carbonic anhydrase in the kidneys of these animals is similar to the distribution in the human kidney Thus the rat and human kidneys are histochemically similar (Hausler 1958 Lonnerholm 1971) except for the straight part of the proximal tubule which always contains detectable enzyme in the rat kidney but sometimes not in the human kidney Also the dog kidney studied by biochemical techniques after microdissection exhibited a similar enzyme pattern as the human kidney (Mattenheimer *et al* 1964 Mattenheimer and Pollak 1965) The renal response to carbonic anhydrase inhibitors is qualitatively the same in man dog and rat (see Maren 1969) These similarities between man dog and rat would justify a discussion of the results of the present study in relation to data obtained by micropuncture techniques in the rat and dog

According to present concepts carbonic anhydrase is present inside the proximal and distal tubular cells where it provides a steady intracellular supply of hydrogen ions by catalyzing the hydration of carbon dioxide (Pitts 1968 Rector 1971) From micropuncture experiments it has also been concluded that enzyme is present in

the brush border of the proximal tubular cells where it would be in functional con tact with the tubular fluid Its role there would be to catalyze the dehydration of carbonic acid, thereby preventing accumulation of excess carbonic acid in the tubular fluid and the generation of steep pH gradients (Rector *et al* 1965) Another function of this membrane bound enzyme could be to facilitate the transport of carbon dioxide across the luminal membrane according to the theory of Enns (1967) This function might be physiologically significant as data recently pre sented by Karlmark (1972) suggest a carbon dioxide gradient across the luminal membrane of the proximal tubular cells

The present study shows that the *convoluted part of the proximal and distal tubules* contain abundant enzyme These cells were stained all over the cytoplasm (except for the apical region of the cells of the proximal tubule) The enzyme ap parently is localized also in the brush border region of the cells of the proximal tubule but it is not possible by the light microscope to decide if the enzyme is located on the inner or the outer (= luminal) side of the brush border membrane

In the rat kidney it has been demonstrated that some bicarbonate is reabsorbed in the loop of Henle *i e* between the end of the proximal convolution and the beginning of the distal convolution (Rector 1971 Malnic *et al* 1972)

In the human kidney the *straight part of the proximal tubule* contains little or no enzyme This part is at present inaccessible for micropuncture studies and little is known about its precise function The marked difference in carbonic anhydrase activity between the convoluted and straight parts of the proximal tubule however would indicate a functional differentiation In a recent study Grantham *et al* (1971) reported that net fluid absorption of isolated rabbit proximal tubules was consider ably less in the straight part than in the convoluted part Al o in the electron microscope the cells from these two parts appear different The cells in the straight part show less extensive lateral and basal cell interdigitations and less mitochondria (Tisher *et al* 1966) structures thought to be associated with transport of electrolytes Certain enzymes i e alkaline phosphatase and glucose 6 phosphatase have also been demonstrated to have different distribution in the two parts of the proximal tubule (see Wajcner 1968)

In the *thin limb* the histochemical picture is difficult to interpret

The *straight part of the distal tubule* however contains enzyme and consequently excretion of hydrogen ions furnished by carbonic anhydrase could take place in this part of the loop of Henle

Many cells in the *collecting duct* contain carbonic anhydrase which could take part in the final adjustment of urinary pH thought to occur in these tubules (Ullrich and Eigler 1958) The most distal part of the collecting ducts close to the papilla lacks enzyme however which suggests that no further acidification takes place in the very tip of the papilla

Finally it should be pointed out that no observations indicate any difference in the distribution of carbonic anhydrase between superficial and deep (= juxtamedul lary) nephrons in the human kidney

Thus the present study shows that the enzyme is present in those parts of the kidney tubules where there is a postulated role for it

Chief cells and intercalated cells in the collecting duct

Light and electron microscopic investigations of the human kidney have shown that the initial segment as well as more distal segments of the collecting duct consists of two types of cells Numerous cells with pale cytoplasm and few organelles named light or chief cells are interspersed with cells having a denser cytoplasm and more numerous organelles dark or intercalated cells (Ericsson *et al* 1965, Myers *et al* 1966 Tisher *et al* 1968) Some cells appear to be intermediate between the chief and intercalated cells The intercalated cells are frequent in the initial and medullary ray segments where they are found singly or in groups of two or three They are less frequent in the outer medullary segment, and they are rarely seen beyond this segment (Myers *et al* 1966) These authors suggested that the intercalated cells merely represent a different state of the chief cells and that they might be derived from them

In the present study some tubules in the cortical labyrinth demonstrated a mosaic of heavily stained unstained or weakly stained cells The tubules could not be identified with certainty but probably are identical with the initial segment of the collecting duct since this is the part of the kidney tubules which contains a mosaic of cells with different morphological characteristics as described above The occasional observation of a confluence of such tubules with mosaic appearance would also identify them as collecting ducts The medullary ray and outer medullary segments of the collecting duct also contained stained and unstained cells In the inner medullary zone however the stained cells disappeared towards the papilla

It was not possible to decide if the heavily stained cells in the collecting duct represented chief cells or intercalated cells Matching pairs of sections stained for carbonic anhydrase and mitochondria was tried but preservation of cellular structure was not good enough to permit matching of single cells in the sections The distribution of the stained and unstained cells did not give any clue as to their identity

The intercalated or dark cells have been recognized in the collecting ducts in the kidney of several mammalian species (Yoshimura and Nemoto 1963) and many different theories as to their function have been presented For example the intercalated cells have been associated with potassium secretion (Kennedy and Parker 1963) liberation of hyaluronidase (Hancox and Komender 1963) and exchange of sodium for hydrogen ions and secretion of ammonia (Young and Wissig 1964) In a recent series of studies it was demonstrated that the number of intercalated cells per cross section of distal tubule or collecting duct in the rat kidney increased when bicarbonate reabsorption was caused to increase (Hagege *et al* 1968 1969 Richet *et al* 1970) Furthermore many chief cells were modified and showed some of the characteristics of intercalated cells The authors believed that these changes which took place within 5 h were due to a functional transformation of the cells and concluded that intercalated and chief cells are two different functional forms of the same cell

These observations make it tempting to speculate that the number of cells in the collecting duct containing carbonic anhydrase may be linked to the need to secrete hydrogen ions This raises the whole question of the regulation of carbonic anhydrase in the kidney That a regulation of carbonic anhydrase activity can occur is suggested by the findings that the enzyme activity of some tissues can vary due to hormonal influence Thus it has been claimed that aldosterone influences carbonic anhydrase activity in the kidney but opposite changes were seen in mouse and rat (Suzuki and Ogawa 1971) In the uterus the enzyme is highly influenced by estrogen and progesterone (see Maren 1967) Thyroid hormone has a marked influence on the level of human erythrocyte isoenzyme carbonic anhydrase B without any clear effect on the other isoenzyme C (Weatherall and McIntyre 1967) It would be interesting to study if the number of enzyme containing cells or the level of enzyme activity in certain regions of the kidney is related to physiological needs

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Acta physiol scand 1973 88 469—480

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Potentiation of Dextran-Induced Histamine Release from Rat Mast Cells by Phosphatidyl Serine

By

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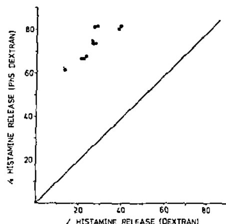
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Abstract

CHAKRAVARTY N A GOTH and P SEN *Potentiation of dextran induced histamine release from rat mast cells by phosphatidyl serine* Acta physiol scand 1973 *88* 469—480

Phosphatidyl serine in 1—50 μg/ml concentrations markedly potentiates histamine release induced by dextran *in vitro* from the mixed peritoneal cells of rats The release occurs within one half minute thus resembling the anaphylactic histamine release The histamine release from isolated pure populations of mast cells which generally respond poorly to dextran was largely restored by phosphatidyl serine The media, employed for the isolation of mast cells viz serum albumin Ficol and acacia were found to inhibit histamine release The albumin inhibition could be overcome by phopshatidyl serine which enhanced the release in spite of the presence of albumin Serum albumin was found to bind phospholipids an effect which may be related to the inhibition of histamine release caused by albumin The effect of some inhibitors of the ATPase system was studied Ouabain in 1—10 mM concentration neither affected the dextran induced histamine release nor its potentiation by phopshatidyl serine Ethacrynic acid (1 mM) had some inhibitory effect on histamine release by dextran alone and dextran + phosphatidyl serine Oligomycin (1 μg/ml) had very little effect on histamine release induced by dextran alone but completely blocked the potentiation of histamine release caused by phosphatidyl serine

Although dextran readily releases histamine from tissues (Halpern 1956) and peritoneal cells (Dias Da Silva and Lemos Fernandes 1965) of rats isolated pure populations of mast cells were found to be refractory to dextran or to show a poor response to it (Lagunoff and Benditt 1960 Chakravarty 1967) Recently phosphatidyl serine has been found to enhance dextran induced and anaphylactic histamine release from mixed rat peritoneal cells (Goth *et al* 1971) The present findings show that phosphatidyl serine can also restore the property of dextran to release histamine from isolated pure populations of mast cells The experiments reported below were undertaken to explore the mechanism of potentiation of dextran induced histamine release by phosphatidyl serine from mixed peritoneal cells and isolated mast cells of rats



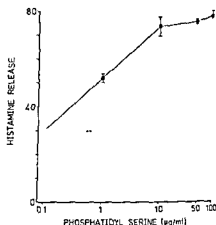


Fig 1 The Figure shows the degree of potentiation of histamine release by phosphatidyl serine (PhS) 50 µg/ml at different levels of the release induced by dextran alone Each point represents histamine release from mixed peritoneal cells *from 1—2 rats*

Fig 2 Dose response curve showing potentiation of dextran induced histamine release from mixed peritoneal cells by phosphatidyl serine Results (mean ± S E) from 4 expts are shown The dotted line represents histamine release by dextran *in the absence of phosphatidyl* serine (30 ± 2 23 per cent)

Material and Methods

Male Wistar rats 300—450 g were used for the experiments Peritoneal cell suspensions from 1—2 rats were prepared as described previously (Chakravarty 1968) They were usually divided into 10—12 samples prewarmed to 37 C in Krebs Ringer solution containing 0 5 *mg/ml* human serum albumin and the incubation was thereafter continued with dextran 1 mg/ml for another 10—12 min The reaction was terminated by placing the samples in ice cold water *Phosphatidyl serine (usually 50 µg/ml) or other substances were added to the cell suspensions* immediately before they were placed in the bath Adequate controls were included and the spontaneous release values (usually 1—? per cent) have been deducted from the results presented *In experiments in which pure populations of mast cells were used the mast cells* were isolated by differential centrifugation in concentrated human or bovine serum albumin as described previously (Chakravarty and Zeuthen 1965 Chakravarty 1965) 94—100 per cent *(average 97 4 per cent) pure mast cells were obtained in this way After washing* the cells were incubated in the same way as in case of mixed peritoneal cells in Krebs Ringer solution containing 1 mg/ml human serum albumin Histamine was assayed by the fluorometric method *(Shore et al* 1959)

Equilibration dialysis experiments for determining the binding of phospholipids to serum albumin were run at 0—4 C Serum albumin dissolved in Krebs Ringer solution was *placed* inside the dialysis bag and ^{14}C phospholipids were added to Krebs-Ringer solution (pH 7 ?) outside the dialysis bag Equilibration was attained within 72 h The radioactivity in the solutions inside and outside the dialysis bag were counted in Aquasol

Phosphatidyl L serine was obtained from *Supelco Inc* (98 per cent pure) and *Schwarz/Mann* (9? per cent pure)—the latter preparation being generally used Phosphatidic acid was obtained from *Supelco Inc* and L serine from *Schwarz/Mann* *Phosphatidyl ethanolamine* 1 2 ^{14}C and phosphatidyl choline 1 2 ^{14}C were purchased from *ICN Chemical & Radioisotope Division* and phosphatidyl inositol ^{14}C (U) and Aquasol from *NEN Chemicals GmbH* Oligomycin a mixture of approx 15 per *cent oligomycin* A *and 85 per cent oligomycin* B was purchased *from Sigma Chemical Co* Dextran (*Pharmacia*) having a mol wt of 80 000 was used for inducing histamine release In diafiltration experiments dextran H^3 mol wt 15 000—17 000 (*NEN Chemicals GmbH*) *was used to determine if it* was bound to other macromolecular substances For isolation of mast cells human serum albumin (*Kabi Stockholm*) or bovine serum albumin (Miles Seravac) were used Ficoll (*Pharmacia*) was dialyzed with distilled water freeze-dried and dissolved *in* 0 9 % NaCl with 10 per cent (v/v) 0 1 M phosphate buffer pH 7 4 and 1 mg/ml serum albumin

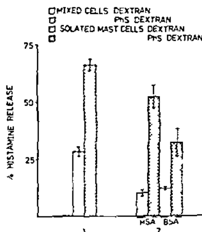


Fig 3 Effect of phosphatidyl serine (PhS) 50 µg/ml on histamine release from pure populations of rat peritoneal mast cells No 1 Histamine release from mixed peritoneal cells Mean±S E of 12 samples No 2 In these expts the peritoneal cells were divided into 2 parts and the mast cells were isolated from one *part in human serum albumin (HSA) and from the* other part in bovine serum albumin (BSA) Mean± S E of 6 samples shown

Results

Potentiation of histamine release induced by dextran from mixed peritoneal cells

Fig 1 shows the potentiating effect of phosphatidyl l serine on histamine release from mixed peritoneal cells induced by dextran Phosphatidic acid and l serine were found to be ineffective It may be seen that the potentiation of dextran induced histamine release by phosphatidyl serine is more pronounced at lower levels of histamine release induced by dextran alone than at higher levels The variation in the response of the cells from different rats to dextran alone was high ranging from 10 to 60 per cent histamine release In presence of phosphatidyl serine however the release was more uniform but still varying from about 50 to 85 per cent We have also come across a poorly reacting batch of rats in which the potentiation has been less pronounced (see below)

The dose response curve for phosphatidyl serine induced potentiation of histamine release is shown in Fig 2 In comparison to the release by dextran alone (dotted line) 1 µg/ml phosphatidyl serine caused a significant enhancement of histamine release The curve is rather steep upto 10 µg/ml phosphatidyl serine and becomes relatively flat thereafter upto 100 µg/ml

The time course of histamine release by dextran alone and with phosphatidyl serine was found to be the same In both cases histamine release was completed with in 1/2 min Dextran induced histamine release with or without phosphatidyl serine thus resembles the explosive release seen in anaphylactic reaction or with compound 48/80 (Bloom and Chakravarty 1970 Bloom *et al* 1967)

If the potentiating effect of phosphatidyl serine is preceded by its uptake by the cells it is likely that the effect would persist after washing the cells In the experiments shown in Table I a part of the peritoneal cell was incubated with phosphatidyl serine for 10—30 min at 37° C and thereafter washed twice prior to incubation with dextran The values were compared with histamine release induced

TABLE I Persistent effect of phosphatidyl serine

Expt no	Time of Incubation with PhS (Min)	% Histamine Release		
		Dextran (A)	PhS+Dextran (B)	PhS → Washed → Dextran (C)
1	10	6 8		11 2
2	20	13 1	51 0	20 7
3	20	19 8	58 8	29 5
4	20	21 9	62 1	30 7
5	30	28 9	61 8	41 8
6	30	38 5	60 7	45 6
Mean		21 5	58 9	29 9

PhS Phosphatidyl Serine Conc 50 μg/ml —
$P < 0\ 001$ by t test for paired comparison between A and C

TABLE II Inhibition of dextran induced histamine release by serum albumin acacia ficoll and caprylic acid

Inhibitor	Conc. (% w/v or mM)	Number of expts	% Histamine Release (Mean ± S E)		P
			Without Inhibitor	With Inhibitor	
Human Serum Albumin	0 5	7	29 3 ± 3 21	16 8 ± 1 74	0 005
Human Serum Albumin	2 %	5	26 3 ± 2 83	10 3 ± 1 73	< 0 005
Bovine Serum Albumin	0 5 %	4	23 6 ± 4 38	3 3 ± 1 22	0 005
Bovine Serum Albumin	2	3	25 7 ± 5 41	2 2 ± 0 79	< 0 025
Acacia	0 5	4	22 8 ± 1 90	16 2 ± 1 50	< 0 05
Acacia	2 %	1	24 2 ± 2 47	4 8 ± 0 51	0 001
Ficoll	0 5 %	4	21 7 ± 3 30	18 1 ± 1 20	0 4
Ficoll	2	3	31 8 ± 1 80	10 7 ± 1 95	0 005
Caprylic acid Na salt	1 mM	1	19 7 ± 0 60	20 0 ± 0 87	0 5
Caprylic acid Na salt	10 mM	1	16 8 ± 1 52	9 6 ± 0 72	0 01

Mean ± S E of 4 samples for each conc of caprylic acid and for 2 % acacia

by dextran alone and by dextran in presence of phosphatidyl serine in the incubation medium It may be seen that the release of histamine was about 40 per cent higher from the cells which were washed after the exposure to phosphatidyl serine The potentiation was however more pronounced where phosphatidyl serine was present during the exposure to dextran

Histamine release induced by dextran from pure populations of mast cells

Histamine release from isolated rat peritoneal mast cells was generally poor in comparison to the release from mixed peritoneal cells as shown in Fig 3 Phosphatidyl serine added in the bath caused a pronounced enhancement of histamine release

TABLE III Effect of pretreatment of peritoneal cells with serum albumin ficoll and caprylic acid on subsequent dextran induced histamine release after washing the cells

Expt no	Pretreatment	Histamine Release		
		Without Pretreatment	With Pretreatment	P
1	HSA	18 7±1 07	17 5±1 15	> 0 4
2	BSA Prepn I Batch I	18 1±1 69	16 1±1 45	> 0 2
3	BSA Prepn I Batch I	23 2±1 55	20 4±1 42	> 0 2
4	BSA Prepn I Batch II	17 9±2 12	12 6±0 46	< 0 05
5	BSA Prepn I Batch II	23 3±1 99	12 6±0 58	< 0 005
6	BSA Prepn II	14 5±0 91	10 6±0 15	< 0 01
7	BSA Prepn III	18 4±0 69	10 0±0 54	< 0 001
8	BSA Prepn IV	17 5±0 72	14 9±1 13	> 0 05
9	Ficoll 30 (w/v)	23 5	19 4	< 0 01
10	Caprylic acid 50 mM	17 4±0 68 —	19 1±0 90	> 0 1
11	*Caprylic acid 100 mM*	*17 4±0 68*	*20 0±0 8*	*< 0 025*

HSA Human Serum Albumin 30 % (w/v)
BSA Bovine Serum Albumin 30 % (w/v)
Preparation I Miles Laboratories Inc Fr V sol
Preparation II Poviet Producten for tube test
Preparation III Poviet Producten for sl de test
Preparation IV Nutritional Biochemicals Corporation (Fraction V unesterified fatty acid poor form)
Histamine release Mean±S E of 4 samples for all except no 9 for which mean values from 6 expts are shown P for no 9 has been calculated by paired comparison

from isolated mast cells The enhanced release from the isolated mast cells however was not as high as the phosphatidyl serine potentiated histamine release from the mixed cells by dextran Continuous exposure to phosphatidyl serine did not give better results than addition of the phospholipid to the incubation medium Using two types of serum albumin in the same experiment it was found that phosphatidyl serine (added in the bath) caused a higher potentiation of histamine release from mast cells isolated in human serum albumin than from those isolated in bovine serum albumin (Fig 3 no 2)

While the results shown in Fig 3 represent the more common type of response of Wistar rat mast cells to dextran we have come across rats responding poorly to dextran *ie* average histamine release 9 % from mixed peritoneal cells and 2 5 % from the isolated mast cells In this group the addition of phosphatidyl serine 50 μg/ml to the isolated mast cells prior to their exposure to dextran raised the histamine release only to 6 % A more marked enhancement of histamine release (to about 17 %) was obtained in this batch of rats by continuous exposure of the cells *to phosphatidyl serine—the phospholipid being added to the solution injected into* peritoneal cavity and thereafter to all the solutions till the incubation of the cells with dextran

TABLE IV Effect of phosphatidyl serine on the albumin inhibition of dextran induced histamine release

Expt no	Inhibitor	% Histamine Release			
		Dextran (A)	Inhibitor+ Dextran (B)	PhS+ Dextran (C)	Inhibitor+ PhS+Dextran (D)
1	HSA 0 5 %	25 8	17 2	67 1	65 0
2	HSA 0 5 %	29 3	22 1	76 9	75 8
3	HSA 0 8 %	11 3	4 3	56 9	44 8
4	HSA 0 8 %	31 1	26 3	82 1	82 1

PhS Phosphatidyl Serine Conc 50 μg/ml
HSA Human Serum Albumin Conc w/v
The difference between columns A and B is significant ($P < 0\ 005$) and that between C and D is not significant ($P > 0\ 2$) by t test for paired comparison

Effect of serum albumin on dextran induced histamine release from mixed peritoneal cells

As seen in Table II both human and bovine serum albumin in 0 5—2 per cent concentration inhibited histamine release the inhibition by bovine serum albumin being much more pronounced As serum albumin may contain unesterified fatty acids the effect of caprylic acid (octanoic acid) was studied It may be seen that 10 mM caprylic acid produced 43 per cent inhibition of histamine release while in 1 mM concentration it had no effect Both human and bovine serum albumin in 2 per cent concentrations caused a more pronounced inhibition of histamine release than was obtained with 10 mM caprylic acid Ficoll (Uvnas and Thon 1959) and acacia (Sugiyama 1971) have also been used for the isolation of mast cells The effect of these macromolecular polysaccharides on histamine release was therefore studied It may be seen from Table II that both acacia and Ficoll in 2 per cent concentration inhibit histamine release Acacia has a more pronounced effect and inhibits even in 0 5 per cent concentration

The possibility that the effect of the macromolecular substances serum albumin acacia and Ficoll might be caused by a binding with dextran was investigated Human and bovine serum albumin (mol wt 67 000) acacia (mol wt 240 000) and Ficoll (mol wt 400 000) were tested for this effect by diafiltration using dextran H³ mol wt 15 000—17 000 A mixture of dextran H³ and one of the other macromolecular substances was repeatedly filtered through Amicon XM 50 ultrafiltration membranes and diluted with physiological salt solution to constant minimal and maximal volumes The filter having a cut-off at 50 000 mol wt allowed dextran H³ to pass through but retained the larger molecules By following the counts in the filtrates it was thus possible to determine if dextran was bound to the larger molecules (serum albumin acacia and Ficoll) It was evident from the curves that no such binding occurred

TABLE V Binding of phospholipids to serum albumin

Expt no	Phospholipid	Albumin (0 5 % w/v)	Phospholipid Binding to Albumin		
			Free (×10⁻ μM)	Bound (×10 μM	Binding
1	PhC	HSA	87 7	43 2	33
2	PhC	HSA	21 9	13 5	36
3	PhC	HSA	22 2	18 6	46
4	PhE	HSA	45 0	51 0	53
5	PhE	HSA	18 8	27 6	59
6	PhE	BSA	14 0	34 6	71
7	PhI	HSA	0 72	3 28	82
8	PhI	HSA	0 57	3 80	8
9	PhI	BSA	0 93	4 27	82
10	PhI	BSA	1 13	4 55	80

HSA Human Serum Albumin BSA Bovine Serum Albumin
PhC = Phosphatidyl Choline PhE — Phosphatidyl Ethanolamine
PhI = Phosphatidyl Inositol

The experiments reported in Table II were performed with the inhibitors present during the incubation with dextran Since the macromolecular substances used for the isolation of mast cells are removed by washing the experiments reported in Table III were performed In these experiments mixed peritoneal cell suspensions were exposed to 30 per cent serum albumin at 0—4° C and to 30 per cent Ficoll or 50—100 mM caprylic acid at room temperature (23° C) for 15 minutes Albumin Ficoll or caprylic acid was then removed by washing and the cells incubated with dextran. Controls were similarly treated in the absence of the substances tested Exposure to human serum albumin did not influence subsequent histamine release by dextran (Expt 1) The effect of bovine serum albumin from different commercial sources differed when tested in this way Of the 4 preparations of bovine serum al bumin used two caused inhibition of histamine release (Expts 6 and 7) while one was without effect (Expt 8) Another preparation had a variable effect of the two batches tested one caused inhibition while the other had no significant effect (Expts 2—5) Cells exposed to Ficoll in a similar manner also showed some reduc tion in histamine release (Expt 9) Caprylic acid in 50 mM concentration when tested in this way had no effect while 100 mM slightly increased the release It may be pointed out in this connection that bovine serum albumin preparations II and III contained caprylic acid amounting to about 100 mM concentration in a 30 per cent albumin solution

The experiments reported in Table IV were performed to determine if the al bumin inhibition of histamine release may be counteracted by phosphatidyl serine It may be seen that phosphatidyl serine was as effective in potentiating histamine re lease in presence of albumin as in its absence Expt 3 particularly shows that in spite of 62 per cent inhibition of histamine release by serum albumin phosphatidyl serine

TABLE IV Effect of phosphatidyl serine on the albumin inhibition of dextran-induced histamine release

Exp. no	Inhibitor	Histamine Release			
		Dextran (A)	Inhibitor – Dextran (B)	PhS – Dextran (C)	Inhibitor – PhS – Dextran (D)
1	HSA, 0.5	25.8	17.2	67.1	65.0
2	HSA, 0.5 *	29.3	22.1	76.9	75.8
3	HSA, 0.8 *	11.3	4.3	59.9	44.8
4	HSA, 0.8 *	31.1	26.3	89.1	89.1

PhS Phosphatidyl Serine, Conc. 50 μg/ml.
HSA Human Serum Albumin, Conc. w/v
The difference between columns A and B is significant ($P < 0.005$) and that between C and D is not significant ($P > 0.2$) by t test for paired comparison.

Effect of serum albumin on dextran induced histamine release from mixed peritoneal cells

As seen in Table II both human and bovine serum albumin in 0.5—2 per cent concentration inhibited histamine release, the inhibition by bovine serum albumin being much more pronounced. As serum albumin may contain unesterified fatty acids, the effect of caprylic acid (octanoic acid) was studied. It may be seen that 10 mM caprylic acid produced 43 per cent inhibition of histamine release while in 1 mM concentration it had no effect. Both human and bovine serum albumin in 2 per cent concentrations caused a more pronounced inhibition of histamine release than was obtained with 10 mM caprylic acid. Ficoll (Lyngås and Thon 1959) and acacia (Sugiyama 1971) have also been used for the isolation of mast cells. The effect of these macromolecular polysaccharides on histamine release was therefore studied. It may be seen from Table II that both acacia and Ficoll in 2 per cent concentration inhibit histamine release. Acacia has a more pronounced effect and inhibits even in 0.5 per cent concentration.

The possibility that the effect of the macromolecular substances serum albumin, acacia and Ficoll might be caused by a binding with dextran was investigated. Human and bovine serum albumin (mol. wt. 67 000), acacia (mol. wt. 240 000) and Ficoll (mol. wt. 400 000) were tested for this effect by diafiltration using dextran H (mol. wt. 15 000—17 000). A mixture of dextran H and one of the other macromolecular substances was repeatedly filtered through Amicon XM 50 ultrafiltration membranes and diluted with physiological salt solution to constant minimal and maximal volumes. The filter having a "cut-off" at 50 000 mol. wt. allowed dextran H[3] to pass through but retained the larger molecules. By following the counts in the filtrates it was thus possible to determine if dextran was bound to the larger molecules (serum albumin, acacia and Ficoll). It was evident from the curves that no such binding occurred.

by phosphatidyl serine was completely blocked Ethacrynic acid 1 mM caused 47 per cent inhibition of histamine release by dextran alone but the inhibitory effect on the potentiation of the release was much more pronounced Ruthenium red 5×10^{-5} M neither affected the histamine release by dextran nor its potentiation by phosphatidyl serine

Discussion

The present findings provide additional evidence for the potentiating effect of phosphatidyl serine on dextran induced histamine release from rat mast cells Marked potentiation is observed with 1—10 μg/ml phosphatidyl serine The time course of the potentiated histamine release and the release by dextran alone is the same—both being complete within one half minute This would be expected if phosphatidyl serine participates in the release process induced by dextran and if the dextran induced histamine release resembles anaphylactic histamine release which is also complete in 20—30 s (Bloom and Chakravarty 1970)

It is evident from the observations on the isolated pure populations of mast cells that dextran acts directly on the mast cells and not through the intermediary of other cells as proposed earlier by Lagunoff and Benditt (1960) However the histamine release induced by dextran alone from isolated mast cells is often poor in comparison to the release from the mixed peritoneal cells Addition of phosphatidyl serine to the isolated mast cells enhances the histamine release induced by dextran and the enhanced release may be higher than the release from the mixed cells in response to dextran alone but is generally lower than the release from the mixed cells in response to dextran+phosphatidyl serine (Fig 3) Obviously some change occurs in the pure mast cells during the process of isolation It was found that the agents used for the isolation of mast cells viz serum albumin Ficoll and acacia in 2 per cent or less concentration inhibit histamine release when they are present in the medium during the exposure of the cells to dextran Diafiltration experiments excluded the possibility that they may block the dextran effect by directly combining with the latter It is likely that these large molecules bind to cell membrane and interfere with the dextran effect in a nonspecific manner

When mixed peritoneal cells were treated with the same concentration of serum albumin as used for the isolation of the mast cell the effect of albumin varied with different preparations and even with different batches Some preparations were found to cause inhibition of histamine release by this method that is after exposing the cells to 30 per cent serum albumin removing the albumin by washing and finally incubating with dextran The possibility that free fatty acids bound to albumin might exert an inhibitory effect on histamine release was considered When the cells were pretreated with caprylic acid (50—100 mM) washed and exposed to dextran the histamine release was not reduced Caprylic acid (10 mM) when present during the exposure of the cells to dextran however had an inhibitory effect on histamine release but the degree of inhibition was lower than the effect of 2 per cent albumin

As the effect of caprylic acid disappears on washing it seems unlikely that the fatty acids bound to albumin could explain the relative resistance of the isolated mast cells to dextran Albumin also has the ability to bind calcium (Carr 1955) It may be a contributory factor for the albumin inhibition of histamine release since calcium is required for the release (Beraldo *et al* 1962) This explanation however seems to be unlikely when the cells are washed with a solution containing calcium before exposure to dextran unless the albumin treatment alters the calcium uptake mechanism

Using ^{14}C marked compounds it is shown that all the 3 phospholipids studied i.e. phosphatidyl choline phosphatidyl ethanolamine and phosphatidyl inositol readily bind to serum albumin (Table V) The binding of phosphatidyl serine to serum albumin has also been reported (Therriault 1964) It is possible that albumin by binding to phospholipids on the membrane may inactivate them or promote their exchange with the medium This view is consistent with the observation that the albumin inhibition of histamine release could be reversed by phosphatidyl serine In fact phosphatidyl serine exerted the usual potentiating effect in spite of the presence of inhibitory concentrations of albumin (Table IV) This is in contrast to the effect of other inhibitors studied which completely blocked the effect of phosphatidyl serine (Table VI)

An exchange of phospholipids between tisues and the medium has been reported (Peterson and Rubin 1969 Siekevitz 1972) It is thus conceivable that added phospholipids are taken up by the mast cells Part of the potentiating effect of phospholipids was found to persist even after washing the cells (Table I) This would be in agreement with an uptake of phospholipids from the medium

Several workers have reported on the activation of the ATPase system by phospholipids (Tanaka and Abood 1964 Fenster and Copenhaver 1967 Wheeler and Whittam 1970 Meissner and Fleischer 1972) It was therefore of interest to study the effect of inhibitors of ATPase activity on the potentiation of histamine release caused by phosphatidyl serine Of the 4 inhibitors used ouabain (1—10 mM) and ruthenium red (5×10^{6} M) had no effect on dextran induced histamine release or on its potentiation by phosphatidyl serine Ouabain is an well known inhibitor of Na K ATP ase (Skou 1957 Bonting 1970) Although Na K ATPase from rat is much less sensitive to ouabain than from other species (Allen and Schwartz 1969) the lack of its effect in high concentrations suggests that Na K ATPase may not be involved in the potentiation of dextran induced histamine release The inorganic dye ruthenium red (10 M) has been shown to cause 50 per cent inhibition of Ca^{++} ATPase of erythrocyte membrane preparations without significantly affecting Na K ATPase or Mg^{++} ATPase (Watson *et al* 1971) It is difficult to draw any conclusion from the negative result of the present experiments with the low concentration of the dye used Higher concentrations caused spontaneous histamine release apparently because of its binding to acid mucopolysaccharides and thus to heparin in the mast cells (Luft 1964 Chakravarty *et al* 1967)

Ethacrynic acid, which inhibits both Na K ATPase and Ca ATPase (Davis

1970 Shami and adde 1971) reduced both the histamine release by dextran and the enhanced relase by dextran+phosphatidyl serine

Oligomycin influenced the histamine release in a characteristic manner A concentration of 1 μg/ml had very little inhibitory effect on histamine release induced by dextran alone but completely blocked the potentiation caused by phosphatidyl serine Oligomycin inhibits Na ATPase (Heinz 1967) but the involvement of this enzyme seems unlikely because of the lack of effect of ouabain on the dextran induced histamine release with or without phosphatidyl serine

Oligomycin sensitive mitochondrial ATPase has been shown to be dependent on Mg^{++} which may be replaced by Mn but not Ca (Tzagoloff 1971) The specific cation effect may possibly vary in the enzyme from different sources Using degraded submitochondrial particles it has been shown that phospholipid is one of the components required for the reconstitution of oligomycin sensitive ATPase (Racker 1969) This may have relevance to our finding that phosphatidyl serine potentiates dextran induced histamine release and oligomycin inhibits the potentiation

Oligomycin also blocks ATP synthesis (Lardy *et al* 1958) Apparently histamine release induced by compound 48/80 is inhibited by this mechanism by oligomycin in higher concentrations (3—5 μg/ml for 50 per cent inhibition) from mixed peritoneal cells (Chakravarty 1967) and in much lower concentrations from isolated mast cells (Johansen and Chakravarty 1972) If the concentration of oligomycin used in the present experiments (1 μg/ml with mixed cells) reduced ATP synthesis in the mast cells the effect must be rather mild—not enough to substantially inhibit the histamine release induced by dextran alone The concentration was however sufficient to completely block the enhancement of the histamine release by phosphatidyl serine A different effect of oligomycin thus seems to be involved here Conceivably the potentiation of histamine release caused by phosphatidyl serine may be related to an oligomycin sensitive ATPase

In view of the reported interaction of phosphatidyl serine and calcium ions (Manery 1966) this mechanism may also contribute to the potentiating effect of phosphatidyl serine on histamine release

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Effects of Increasing Glucose Concentrations on the Glucose Metabolism in Arterial Tissue and Intestinal Smooth Muscle

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Abstract

ARNQVIST H J *Effects of increasing glucose concentrations on the glucose metabolism in arterial tissue and intestinal smooth muscle* Acta physiol scand 1973 *88* 481—490

The effect of increasing glucose concentrations (0—44 4 mM) on the glucose metabolism in bovine mesenteric arteries and rabbit colon smooth muscle was studied *in vitro* The glucose uptake in both these tissues increased when the concentration of glucose in the medium was raised with saturation at higher glucose concentrations The rise in glucose uptake was most pronounced in the range 0—11 1 mM The effect of glucose concentrations on lactate elimination was similar in both tissues to that on glucose uptake Increases in the tissue levels of glycogen and lactate were observed up to a medium concentration of 5 6—11 1 mM glucose A further rise in the medium glucose had no effect on the glycogen and lactate contents In bovine mesenteric arteries the intracellular accumulation of glucose carbon from ^{14}C-labelled glucose did not change significantly when the glucose concentration was raised from 5 6 to 44 4 mM The membrane transport of glucose in bovine mesenteric arteries was characterized by saturation kinetics of the Michaelis-Menten type with a K_m of 4 0 mM These results suggest that the glucose metabolism in bovine mesenteric arteries and rabbit colon smooth muscle is proportionally affected to a greater extent by variations in glucose concentration near the physiological level than by extreme hyperglycemia

There are indications that the metabolism of the vascular wall may have importance for the development of vascular diseases (Adams and Bayliss 1969 Kirk 1968 Whereat 1967) In diabetes mellitus the metabolic disorder is accompanied by an increased frequency of diseases of the vascular system (Schettler and Wahl 1969) It is therefore of interest to study how insulin and the diabetic state influence the metabolism of vascular tissue

In vascular smooth muscle as in other tissues glucose is metabolized to CO_2 lactate glycogen and lipids (Somlyo and Somlyo 1968) When rabbit aorta is incubated in ^{14}C labelled glucose a major proportion of the glucose uptake can be accounted for as lactate while progressively smaller fractions are represented by oxidation to CO_2 glycogenesis and incorporation into total lipid (Mulcahy and Winegrad 1962)

In bovine mesenteric arteries and rabbit colon smooth muscle the membrane transport of monosaccharides has the characteristics of facilitated diffusion (Arnqvist 1972) The membrane transport of glucose seems to be a rate limiting step for the glucose metabolism in these tissues (Arnqvist 1971, 1972) Insulin (0 1 U/ml) stimulates the monosaccharide transport in bovine mesenteric arteries and rabbit colon smooth muscle to a minor degree as compared with skeletal muscle (Arnqvist 1972)

The aim of the present investigation was to study the role of the extracellular glucose concentration for the glucose metabolism in vascular smooth muscle The effect of varying glucose concentrations on glucose uptake, lactate elimination the tissue content of lactate and glycogen and the intracellular accumulation of glucose carbon were examined Bovine mesenteric arteries which are rich in smooth muscle (Ducret 1930) were used As the vascular wall consists only partly of smooth muscle cells the glucose metabolism of a preparation consisting almost exclusively of smooth muscle (the muscle layer of rabbbit colon) was also investigated

Material and methods

Animals Rabbits weighing 2—3 kg were used All animals were starved 20—24 h before the experiments Bovine mesenteric arteries were obtained from a slaughter house

Dissection and incubation The rabbits were killed by a blow on the neck The part of the colon where the taeniae are united and cover one half of the circumference was removed flushed with Krebs-Henseleit bicarbonate buffer at room temperature and cut up longitudinally The mucosal layer was carefully scraped away From the part covered with taenia similarly shaped specimens 3—5 mm broad 10—15 mm long and weighing 30—60 mg were prepared

Bovine mesenteric arteries were dissected out 30—60 min after slaughter A homogeneous 10—15 cm long segment was cut off and transported to the laboratory in Krebs-Henseleit bicarbonate buffer at 37 C the buffer was continuously gassed with a mixture of 95 % O_2 and 5 % CO_2 Cooling of the preparation was avoided as it has been found to disturb the glucose metabolism in vascular tissue (Scott *et al* 1970) The arteries were carefully freed from adventitia cut up longitudinally and divided into 4—5 mm broad and 8—10 mm long pieces weighing 50—100 mg Histological control of the intima media preparation showed that almost all adventitia was removed and no perivascular fat was present

The tissue preparations were incubated in 25 ml flasks containing 4 ml of medium For determination of the glucose uptake 10 ml flasks containing 1 8 or 2 3 ml of medium were used The flasks were gassed with a mixture of 95 O_2 and 5 CO_2 for 20 s sealed with tightly fitting rubber stoppers and kept in an agitation bath at 37 C during the preincubation (10—30 min) and incubation periods

Incubation medium Krebs Henseleit bicarbonate buffer with the following composition was used (mM) 120 NaCl 4 7 KCl 1 3 $CaCl_2$ 1 2 $MgSO_4$ 1 2 KH_2PO_4 25 $NaHCO_3$ The buffer was equilibrated with a mixture of 95 % O_2 and 5 CO_2 at 37 C which gave a pH of 7 4 1 mg glucose/ml was added to the preincubation medium

Determination of glucose uptake The glucose uptake was calculated by determining the glucose concentration in the incubation medium before and after the incubation period Glucose was determined by the hexokinase glucose 6 phosphate dehydrogenase method (Stein 1962) To obtain a measurable glucose uptake it was necessary to pool several pieces of tissue 150—200 mg of rabbit colon smooth muscle was incubated in 1 8 ml of medium and 200—300 mg of bovine mesenteric arteries was incubated in 2 3 ml of medium Before the measurement of the glucose uptake the tissue was incubated for 30 min in a medium with the same glucose concentration This was done to equilibrate the extracellular space with the concentration of glucose in the medium In control experiments without tissue the glucose concentration of the incubation medium did not change during the incubation period

Accumulation of glucose carbon from ^{14}C labelled glucose After incubation in a medium containing uniformly labelled D-glucose ^{14}C the tissue was rinsed in buffer blotted weighed and dissolved in 1 ml Soluene 9 ml of scintillation fluid was added (toluene containing 5 g

PPO and 0.3 g dimethyl POPOP per litre) 100 μl of the incubation medium was dissolved in 10 ml Instagel. Duplicate samples were always taken. Radioactivity was measured in a liquid scintillation spectrometer (Packard Tri Carb). The degree of quenching was determined by the external standard technique. The counting efficiency was 90—95 %. The tissue content of glucose carbon was calculated as μmole glucose equivalents by the equation

$$\frac{\text{cpm/g tissue wet weight}}{\text{cpm/ml incubation medium}} \times \text{medium glucose concentration } (\mu\text{mol/ml}) = \mu\text{mol glucose carbon/g tissue wet weight}$$

Extracellular space. Sorbitol ^{14}C was added to the incubation medium in a final concentration of 0.5 mM. The isotope contents of the tissue and incubation medium were determined as described above. The distribution (space) of sorbitol ^{14}C in whole tissue was calculated as follows

$$\text{distribution } (\%) = \frac{\text{cpm/mg tissue wet weight}}{\text{cpm/}\mu\text{l incubation medium}} \times 100$$

Determination of glucose space. After the incubation the tissue was rinsed in buffer, blotted and frozen at −80 °C in frigen 12 containing solid CO_2. The frozen tissue was homogenized in 6 % perchloric acid (PCA) and neutralized with K_2CO_3. The glucose contents of the tissue extract and incubation medium were determined as described above. The tissue distribution (space) of glucose was calculated by the formula

$$\text{distribution } (\%) = \frac{\text{content in wet tissue } (\mu\text{mol/g})}{\text{medium concentration } (\mu\text{mol/ml})} \times 100$$

Lactate determination. For determination of the tissue content of lactate the tissue samples were rinsed in buffer, blotted and frozen at −80 °C. The tissue was homogenized in 6 % PCA. After neutralization with K_2CO_3 the lactate content of the tissue extract was determined.

Lactate elimination from the tissue was calculated by determining the lactate content of the incubation medium after the incubation period. Lactate in tissue extract and incubation medium was assayed by the lactate dehydrogenase method according to Lundholm *et al.* (1963).

Glycogen determination. The tissue pieces were blotted and rapidly frozen at −80 °C. The tissue was digested in 0.5—1 ml 1 M KOH by heating in boiling water for 60—90 min. The extract was neutralized with HCl and glycogen was determined by the amyloglucosidase method (Keppler and Decker 1970).

Chemicals. Hexokinase, glucose 6 phosphate dehydrogenase, lactate dehydrogenase and amylo-α 1,4 α 1,6 glucosidase for the enzymatic determinations were obtained from Boehringer Mannheim. Instagel and Soluene were commercial preparations of Packard Instrument Company Inc. D glucose ^{14}C (U) and sorbitol ^{14}C were obtained from the Radiochemical Centre, Amersham, England.

Statistical analysis. Mean values are given ± S.E. The significance of differences between samples was determined by means of Student's t test. A difference which gave $p < 0.05$ was considered significant. Linear regression and correlation coefficients were calculated with a Hewlett Packard desktop computer using standard formulas.

Results

Time course of the glucose uptake. From Fig. 1 it appears that the glucose uptake in bovine mesenteric arteries and rabbit colon smooth muscle increased with time for at least 180 min. The glucose uptake showed a linear relationship with time both in bovine mesenteric arteries ($r = 0.93$) and rabbit colon smooth muscle ($r = 0.92$).

Effect of glucose concentration on glucose uptake. The glucose uptake was determined after an incubation time of 180 min. From Fig. 2 it is seen that the glucose uptake in both tissues studied increased when the concentration of glucose in the medium was varied from 2.8 up to 44.4 mM. The increase in glucose uptake was smaller at higher glucose concentrations indicating saturation kinetics. The glucose uptake was higher in rabbit colon smooth muscle than in bovine mesenteric arteries.

It was earlier found that the membrane transport of glucose was rate limiting for the glucose uptake in bovine mesenteric arteries in the concentration range 5.6 to

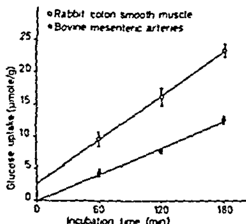


Fig 1

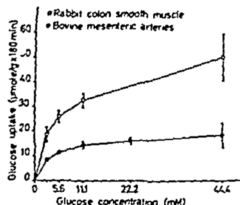


Fig 2

Fig 1 Glucose uptake in bovine mesenteric arteries and rabbit colon smooth muscle determined after incubation periods of 60 120 and 180 min. The concentration of glucose in the medium was 5 6 mM Glucose uptake is expressed as µmol/g tissue wet weight. The lines drawn were obtained by linear regression of the glucose uptake data. Mean ± S E Bovine mesenteric arteries, $n = 8$ Rabbit colon smooth muscle $n = 5$

Fig 2 Effect of glucose concentrations 2 8—44 4 mM on the glucose uptake in bovine mesenteric arteries ($n = 11$) and rabbit colon smooth muscle ($n = 7$) The glucose uptake was determined after an incubation period of 180 min Mean ± S E

44 mM (Arnqvist 1972) The results reported below show that the diffusion of glucose into the extracellular space became rate limiting for the glucose uptake below a medium concentration of 5 6 mM These findings indicate that the glucose uptake in the range 5 6 to 44 4 mM reflects the glucose transport From the data shown in Fig 2 the mean values for the glucose uptake (5 6—44 4 mM) in bovine mesenteric arteries were plotted in a Lineweaver Burk diagram (Lineweaver and Burk 1934) The plot (Fig 3) was linear with a correlation coefficient of 0 99 The regression line obtained ($y = 0\ 204X + 0\ 051$) gave a Km value of 4 0 and Vmax 19 6 µmol/g × 180 min

Effect of glucose concentration on the intracellular accumulation of glucose carbon from ¹⁴C labelled glucose The accumulation of glucose carbon from ^{14}C-labelled glucose in total tissue was determined in bovine mesenteric arteries after an incubation period of 180 min To calculate the amount of glucose carbon accumulated within the cells the glucose in the extracellular space had to be estimated If the membrane transport of glucose is rate limiting for the glucose metabolism there will be no intracellular glucose and the concentration of glucose in the extracellular space will be equal to the concentration in the medium. In bovine mesenteric arteries the tissue content of glucose corresponded to a distribution in the extracellular space as estimated by sorbitol when the concentration of glucose in the medium was varied from 5 6 to 44 4 mM (Arnqvist 1972)

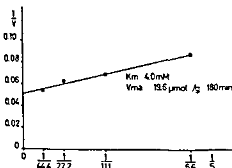


Fig 3

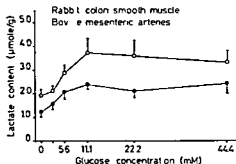


Fig 4

Fig 3 Application of the Michaelis-Menten equation to the glucose transport in bovine mesenteric arteries The reciprocals of the mean values for V (µmol glucose/g × 180 min) are plotted against the reciprocals of S (mM)

Fig 4 Effect of glucose concentration 0—44 4 mM on lactate content of bovine mesenteric arteries (n = 10) and rabbit colon smooth muscle (n = 8) The lactate was determined after an incubation period of 180 min S E is indicated by the length of the vertical bars

At low concentrations in the medium the diffusion of glucose through the extra cellular space may become rate limiting for the glucose metabolism and the concen tration of glucose in the extracellular space will be lower than that in the medium To check this the tissue distribution of glucose was determined in bovine mesenteric arteries after an incubation period of 180 min and compared with the extracellular space as estimated by sorbitol At a medium concentration of 2 8 mM glucose the glucose space was 32 3 ± 2 1 % and the sorbitol space 44 1 ± 1 1 % of the wet tissue weight This difference was significant ($p < 0.01$ n = 5) When the concentration of glucose was 5 6 mM the glucose space was 39 3 ± 2 4 % (n = 9) and the sorbitol space 42 0 ± 0 8 % (n = 9) and the difference was not significant It therefore seems probable that the diffusion of glucose through the extracellular space is rate limiting for the glucose metabolism in bovine mesenteric arteries at concentrations of glucose in the medium lower than 5 6 mM

The intracellular accumulation of glucose carbon was determined in the concen tration range 5 6—44 4 mM by assuming that the extracellular concentration of glucose was equal to that in the medium Sorbitol was used to estimate the extra cellular space The results are shown in Table I The calculated intracellular con tent of glucose carbon was rather variable and did not increase significantly within the studied concentration range The total tissue accumulation of glucose carbon in creased with rising glucose concentrations due to an increased glucose content in the extracellular space

Effect of glucose concentration on lactate content The tissue content of lactate was determined after incubation for 180 min in a medium containing 0—44 4 mM glucose It is evident from Fig 4 that the lactate content of bovine mesenteric arteries and rabbit colon smooth muscle was rather variable In both tissues the

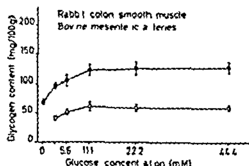


Fig 5 Effect of glucose concentration on glycogen content of bovine mesenteric arteries (n = 11) and rabbit colon smooth muscle (n = 8) The glycogen content was determined after an incubation period of 180 min Mean ± S E

lowest lactate content was found when the incubation medium contained no glucose When glucose was added the tissue content of lactate increased The lactate content of bovine mesenteric arteries was increased at glucose concentrations of 5 6—44 4 mM compared with that in the absence of glucose and the content at 11 1 mM was significantly higher ($p < 0\ 02$) than the value at 2 8 mM In the glucose concentration range 5 6 to 44 4 mM there were no significant differences between the tissue content of lactate at varying glucose concentrations Also in rabbit colon smooth muscle the lactate content was higher in the range 5 6—44 4 mM than in the absence of glucose and the lactate content at 11 1 mM was higher than at 2 8 mM ($p < 0\ 05$)

Effect of glucose concentration on glycogen content The glycogen content was determined after incubation of the tissue for 180 min in a medium containing 2 8—44 4 mM glucose In bovine mesenteric arteries this content was also determined after incubation in the absence of glucose From Fig 5 it appears that the glycogen content in bovine mesenteric arteries and rabbit colon smooth muscle increased with increasing glucose concentrations in the medium up to a concentration of 11 1 mM at which point it reached a plateau It can also be seen from Fig 5 that the glycogen content in bovine mesenteric arteries was higher than in rabbit colon smooth muscle

TABLE I Effect of glucose concentration in the medium on the intracellular accumulation of glucose carbon in bovine mesenteric arteries The accumulation of glucose carbon in whole tissue was determined after an incubation period of 180 min in a medium containing C labelled glucose The tissue content of glucose carbon was calculated as μmol glucose equivalents/g tissue wet weight Extracellular space was measured by sorbitol C (0 5 mM) in the presence of unlabelled glucose Mean ± S E (n = 9)

Medium concentration of glucose (mM)	Accumulation of glucose carbon in total tissue μmol/g	Sorbitol space (of the total tissue weight)	Extracellular glucose carbon (μmol/g)	Intracellular glucose carbon (μmol/g)
5 6	6 05 ± 0 43	43 1 ± 0 9	2 41 ± 0 05	3 66 ± 0 47
11 1	8 50 ± 0 50	40 9 ± 1 0	4 54 ± 0 11	3 96 ± 0 53
22 2	13 63 ± 0 63	41 7 ± 1 0	9 26 ± 0 22	4 37 ± 0 52
44 4	22 02 ± 0 82	41 1 ± 1 1	18 25 ± 0 49	3 74 ± 0 81

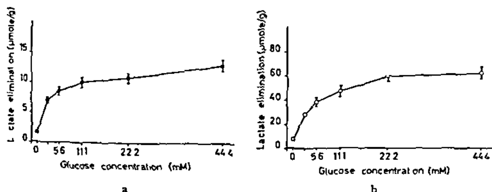


Fig 6 a. Effect of glucose concentration on lactate elimination in bovine mesenteric arteries determined after incubation of the tissue for 180 min in a medium containing 0—44.4 mM glucose Mean±S E (n ~ 7)

Fig 6 b Effect of glucose concentration on lactate elimination in rabbit colon smooth muscle The experimental conditions were the same as in Fig 6 a. Mean±S E (n = 7)

Effect of glucose concentration on lactate elimination Lactate elimination was determined in the two tissues during an incubation period of 180 min in a medium containing 0—44.4 mM glucose (Fig 6 a and b) When no glucose was present in the incubation medium the lactate elimination was low, and in the presence of glucose it rose markedly Up to a concentration of 5.6—11.1 mM the increase was rapid whereafter the curves flattened out indicating saturation kinetics for the lactate production The large increase in lactate elimination in the presence of glucose suggests that the glucose in the medium was the source of most of the lactate The glucose uptake (Fig 2) was higher in rabbit colon smooth muscle than in bovine mesenteric arteries From Fig 6 a and b it is seen that also the lactate elimination was higher in rabbit colon (note that there are different scales on the Y axes) When the values for glucose uptake and lactate elimination at a medium concentration of 5.6 mM glucose are compared it is found that lactate elimination accounted for 36.9 % of the glucose uptake in bovine mesenteric arteries and 75.4 % in rabbit colon smooth muscle

Discussion

The glucose uptake in bovine mesenteric arteries and rabbit colon smooth muscle increased linearly with time during incubation periods of 180 min It was found earlier (Arnqvist 1972) that the extracellular space as estimated by sorbitol remained constant and that L-glucose did not penetrate the cell membrane during incubation periods of this duration It therefore seems probable that these smooth muscle preparations remain intact and can be used for metabolic studies during incubation periods of at least 180 min

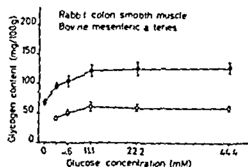


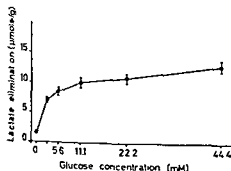
Fig 5 Effect of glucose concentration on glycogen content of bovine mesenteric arteries (n = 11) and rabbit colon smooth muscle (n = 8) The glycogen content was determined after an incubation period of 180 min Mean ± S E

lowest lactate content was found when the incubation medium contained no glucose When glucose was added the tissue content of lactate increased The lactate content of bovine mesenteric arteries was increased at glucose concentrations of 5 6—44 4 mM compared with that in the absence of glucose and the content at 11 1 mM was significantly higher ($p < 0 02$) than the value at 2 8 mM In the glucose concentration range 5 6 to 44 4 mM there were no significant differences between the tissue content of lactate at varying glucose concentrations Also in rabbit colon smooth muscle the lactate content was higher in the range 5 6—44 4 mM than in the absence of glucose and the lactate content at 11 1 mM was higher than at 2 8 mM ($p < 0 05$)

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TABLE 1 *Effect of glucose concentration in the medium on the intracellular accumulation of glucose* carbon in bovine mesenteric arteries The accumulation of glucose carbon in whole tissue was determined after an incubation period of 180 min in a medium containing C labelled glucose The tissue content of glucose carbon was calculated as μmol glucose equivalents/g tissue wet weight Extracellular space was measured by sorbitol C (0 5 mM) in the presence of unlabelled glucose Mean ± S E (n = 9)

Medium concentration of glucose (mM)	Accumulation of glucose carbon in total tissue μmol/g	Sorbitol space (of the total tissue weight)	Extracellular *glucose carbon* (μmol/g)	Intracellular glucose carbon (μmol/g)
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11 1	8 50 ± 0 50	40 9 ± 1 0	4 54 ± 0 11	3 96 ± 0 53
22 2	13 63 ± 0 65	41 7 ± 1 0	9 26 ± 0 22	4 37 ± 0 52
44 4	22 02 ± 0 82	41 1 ± 1 1	18 25 ± 0 49	3 74 ± 0 81



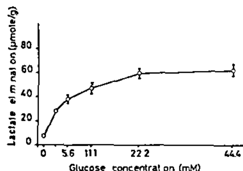


Fig 6a Effect of glucose concentration on lactate elimination in bovine mesenteric arteries determined after incubation of the tissue for 180 min in a medium containing 0—44.4 mM glucose. Mean±S E (n = 7)

Fig 6b Effect of glucose concentration on lactate elimination in rabbit colon smooth muscle The experimental conditions were the same as in Fig 6a. Mean±S E (n = 7)

Effect of glucose concentration on lactate elimination Lactate elimination was determined in the two tissues during an incubation period of 180 min in a medium containing 0—44.4 mM glucose (Fig 6a and b) When no glucose was present in the incubation medium the lactate elimination was low and in the presence of glucose it rose markedly Up to a concentration of 5.6—11.1 mM the increase was rapid whereafter the curves flattened out indicating saturation kinetics for the lactate production The large increase in lactate elimination in the presence of glucose suggests that the glucose in the medium was the source of most of the lactate The glucose uptake (Fig 2) was higher in rabbit colon smooth muscle than in bovine mesenteric arteries From Fig 6a and b it is seen that also the lactate elimination was higher in rabbit colon (note that there are different scales on the Y axes) When the values for glucose uptake and lactate elimination at a medium concentration of 5.6 mM glucose are compared it is found that lactate elimination accounted for 36.9 % of the glucose uptake in bovine mesenteric arteries and 75.4 % in rabbit colon smooth muscle

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The glucose uptake in bovine mesenteric arteries and rabbit colon smooth muscle increased linearly with time during incubation periods of 180 min It was found earlier (Arnqvist 1972) that the extracellular space as estimated by sorbitol remained constant and that L glucose did not penetrate the cell membrane during incubation periods of this duration It therefore seems probable that these smooth muscle preparations remain intact and can be used for metabolic studies during incubation periods of at least 180 min

To study the effect of glucose concentrations on the total utilization of glucose in bovine mesenteric arteries and rabbit colon smooth muscle the glucose uptake was determined at concentrations of 2 8 to 44 4 mM glucose in the medium. In both tissues the glucose uptake increased with increasing medium concentrations of glucose The rise in the glucose uptake was not a linear function of the glucose concentration in the medium but approached a maximum at higher concentrations From Fig 2 it is seen that the effect of varying glucose concentrations on the glucose uptake was most pronounced in the range of 0—11 1 mM Above 11 1 mM the curves flattened out and variations in the glucose concentration had only small effects on the glucose uptake These findings suggest that the total utilization of glucose in bovine mesenteric arteries and rabbit colon smooth muscle is proportionally affected to a greater extent by variations of the glucose concentration near the physiological range than by extensive hyperglycemia

When the membrane transport of glucose is rate limiting for the glucose uptake the velocity of the glucose uptake reflects the glucose transport velocity At glucose concentrations of 5 6 to 44 4 mM in the medium the membrane transport of glucose is rate limiting for the glucose uptake in bovine mesenteric arteries (Arnqvist 1972) The results of this study show that the diffusion of glucose through the extracellular space becomes rate limiting at medium concentrations below 5 6 mM In the concentration range 5 6 to 44 4 mM the glucose uptake in bovine mesenteric arteries may therefore be considered to reflect the membrane transport of glucose A linear relationship was found when the glucose uptake values (5 6—44 4 mM) were plotted in a Lineweaver Burk diagram The membrane transport of glucose in bovine mesenteric arteries is thus characterized by saturation kinetics of the Michaelis Menten type This result agrees with an earlier finding (Arnqvist 1972) that monosaccharides enter smooth muscle cells by facilitated diffusion The Lineweaver Burk plot gave a Km value of 4 0 mM and Vmax 19 6 µmol/g × 180 min In rat skeletal muscle Chaudry and Gould (1969) reported a Km value of 35 mM for the membrane transport of glucose and in rat adipose tissue Crofford and Renold (1965) found Km to be 90 mM Compared with these values the Km for glucose transport in bovine mesenteric arteries was low This finding indicates that the glucose transport system in bovine mesenteric arteries has a higher affinity for glucose than the transport system in rat skeletal muscle and adipose tissue

A high rate of aerobic glycolysis is stated to be a characteristic feature of the glucose metabolism in arterial tissue (Lehninger 1959) Lactate production represented 80 % of the total metabolism of ^{14}C labelled glucose in dog popliteal artery while only 2 % was recovered as CO_2 (Beaconsfield 1963) In rat aorta from 1 year old male rats 54 7 % of ^{14}C labelled glucose was incorporated into lactate and 1 4 % into CO_2 (Newmark et al 1972) Cooling of arterial tissue irreversibly decreased glycolysis with 50 % while respiration was decreased fivefold (Scott et al 1970) In most studies of the carbohydrate metabolism of arterial tissue and also in the studies cited above the experiments have been done on precooled tissue It is therefore possible that the proportion of glucose metabolized to CO_2

should be somewhat higher Only the elimination of lactate from the ti ue w determined in this study At a glucose concentration of 5 6 mM the lactate elimina tion to the medium corresponded to 36 9 % of the gluco e uptake in bovine mesenteric arteries and 75 4 % of the glucose uptake in rabbit colon smooth muscle In both tissues the lactate elimination increased with increasing gluco e co icentra tions At higher glucose concentrations the rise in lactate elimination levelled off in dicating saturation kinetics for the lactate production with increasing concentrations of glucose in the medium As with the glucose uptake the effect of gluco e concen trations was most pronounced in the range 0—11 1 mM In the absence of glucose the lactate elimination was low indicating that the lactate production was dependent to a large extent on the glucose uptake from the medium

The effect of glucose concentrations (5 6—44 4 mM) on the total accumulation of gluco e metabolites in bovine mesenteric arteries was studied by determining the intracellular accumulation of glucose carbon from ^{14}C labelled glucose As there is no intracellular glucose under these conditions (Arnqvist 1972) the glucose carbon represents glucose metabolites accumulated during the incubation period of 180 min The intracellular content of glucose carbon was rather variable and did not change significantly when the medium concentration of glucose was raised from 5 6 to 44 4 mM The tissue content of glucose metabolites was therefore little affected by vari ation of the medium glucose above a concentration of 5 6 mM The same relation ship was reflected in the ti sue content of lactate and glycogen which probably represents the main part of the tissue metabolites of ^{14}C labelled glucose in arterial tissue (Beanconsfield 1963) Glucose up to a concentration of 5 6—11 1 mM in creased the tissue content of lactate and glycogen but a further increase up to 44 4 mM had no significant effect on these parameters The tissue content of lactate and glycogen in rabbit colon smooth muscle was al o influenced by the glucose in the medium up to a concentration of 5 6—11 1 mM As in bovine mesenteric arteries a further rise in the glucose concentration in the medium did not increase the tis ue levels of glycogen and lactate

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Acta physiol scand 1973 88 491—504

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The Liver-like Anion Transport System in Rabbit Kidney, Uvea and Choroid Plexus II Efficiency of Acidic Drugs and Other Anions as Inhibitors

By

ERNST H BARANY

Received 20 October 1972

Abstract

BARANY E H *The liver like anion transport system in rabbit kidney uvea and choroid plexus II Efficiency of acid drugs and other anions as inhibitors* Acta physiol scand 1973 *88* 491—504

In vitro uptake of labelled iodipamide in the presence of a large excess of hippurate is due to a distinct possibly complex anion uptake system the L-system present in kidney cortex ciliary processes and lateral choroid plexus A large number of organic anions of very different structure many of them drugs have now been screened for ability to inhibit the L system in rabbit tissues The L system is depressed much more by cholegraphic than by urographic radio-contrast agents A majority among substances known to be excreted by the liver depressed the L-system Substances of mol wt below 175 have little effect among heavier anions inhibition is common. This size dependence is similar to that for biliary excretion No structural specificity can be discerned In all these respects the L system is l er like Certain liver excreted anions have little effect on the L-system This indicates that the organic anion tran.port system of the liver is composite The affinity of many drugs to the L-system is bound to affect their pharmacokinetic behaviour and can give rise to drug interactions

A liver like anion transport system in kidney cortex anterior uvea and choroid plexus from several species has recently been described (Barany 1972 1973) These tissues accumulate iodipamide *in vitro* despite the presence of sufficient hippurate to saturate the well known hippurate transporting system The present paper reports a rough first screening of a large number of compounds for their ability to depress the uptake of iodipamide in the presence of excess hippurate Such an ability indicates affinity to the liver like system Affinity has been found in many acidic drugs Since it is probable (Barany 1973) that the direction of transport of the system is out of the eye or the cerebrospinal fluid into the blood the affinity of drugs to the new system has obvious pharmacokinetic implications

Three groups of acidic substances were tested The first is simple chemicals with out special biological activity which it was hoped might shed light on the require ments for affinity to the new system The second group is substances with known

excretion routes e g X ray contrast agents The third group consists of drugs or substances with known biological properties and which serve two functions in the present investigation they are chemicals that might illuminate the requirements for affinity and at the same time the correlation between biological activity and affinity to the new system may be interesting as such

In the following the liver like system is called the L system and the classical hippurate transporting one the H system The latter also helps in the transport of iodipamide to a different degree in different tissues and species The L-system may be composite (Barany 1972)

Methods

The animals were pigmented or white rabbits of both sexes The experimental methods solutions etc were as described (Bárány 1972) Briefly slices of kidney cortex pieces of iris + ciliary body = anterior uvea = eye and lateral choroid plexus were incubated for 20—30 min in a K rich medium containing labelled iodipamide in 0 5—1 5 μM chemical concentration Hippurate in excess was always present in the medium

The aim in the majority of experiments was to use the hippurate concentration 10 mM One long series of experiments however was performed with 8 9 mM by mistake These were not discarded Lately the concentration has been raised to 30 mM Since half saturation of the H system occurs at about 0 5 mM hippurate (Fig 3 Bárány 1972) between 6 and 2 per cent of the primary binding sites receptors of that system have been free in the experiments These can also take up iodipamide and because of the high degree of inhibition of this set of receptors their uptake is quite resistant to further inhibition The experiments therefore measure inhibition of the L system but they underestimate it because of a small residual uptake of iodipamide by the H system There is also some evidence that one component of the L system has even less affinity to hippurate than the rest of the system. The uptake figures in the tables can therefore not be converted into affinity figures

For many substances only one experiment was made at each of two concentrations one order of ten apart The lower concentration was obtained by dilution of the higher In the typical case one kidney and one eye were removed from the anesthetized animal and tissue samples run with 3—4 different putative inhibitors at 0 1 mM Then the other kidney the other eye choroid plexus and sometimes liver were obtained and run with the same inhibitors but now at 1 mM There was at least 1 separate control bottle for each run and very often 1 separate totally inhibited bottle with 12 6 mM iodipamide Thus there were separate controls for each of the two concentrations of inhibitor but often a common control bottle for several inhibitors run at the same time Each bottle as a rule contained 2 slices of kidney cortex 1 sector of anterior uvea 1/2 lateral ventricular plexus and occasionally a few slices of liver The average tissue/medium ratio was calculated for each tissue corrected for unspecific uptake in the totally inhibited bottle and divided by the similarly corrected tissue/medium ratio of the corresponding tissue in the control bottle This yielded the percent uptake figures Since there was so little plexus tissue available many inhibitors were not tested on choroid plexus

In order to estimate the range of random variation under our conditions six blank experiments were run with 5 bottles in each Four of the 5 bottles contained identical solutions with 10 mM hippurate but no other inhibitor the fifth bottle contained 12 6 mM iodipamide for total inhibition Kidney cortex slices were incubated exactly as in the standard screening experiment Average specific tissue/medium ratio was 3 87 without hippurate it would have been about 15 In the evaluation the four uninhibited bottles were taken in turn as control bottle and the other three as experimental Thus 12 different percent uptake figures resulted from each experiment a total of 72 The 4 lowest per cent uptake figures found were 70 72 73 and 77

Similarly the 4 lowest per cent uptake figures among 36 found for anterior uveal pieces (average specific T/M = 0 58 without hippurate about 7 5) were 60 62 63 and 70 and the three lowest among 30 for choroid plexus (average specific T/M = 6 15 without hippurate about 13) 48 49 and 53 Thus in round figures if an uptake found with a single control bottle in a single experiment is more than 70 per cent for kidney slices 60 per cent for anterior uvea and 50 per cent for choroid plexus the depression is rather questionable Conversely depressions of up to 30 40 and 50 per cent can be completely hidden if the uptake in

TABLE I Effect of X ray contrast agents on iodipamide uptake

Cholegraphic acids		Uptake percent of controls						Urographic acids		Uptake percent of controls					
		Kidney		Plexus		Eye				Kidney		Plexus		Eye	
No	Millimolarity	0 1	1 0	0 1	1 0	0 1	1 0	No	Millimolarity	0 1	1 0	0 1	1 0	0 1	1 0
1	Iodipamide[1]	37	17	25	8	81	54	11	Iothalamic[4]	101	69			65	58
2	Ioglycamic	52	29			64	61	12	Diatrizoic	101	69		35	105	102
3	Iosefamic[1,2]	81	97			124	96	13	Acetrizoic	101	69		63	147	80
4	*Iopanoic*	*41*	*9*		*18*	*52*	*26*	*14*	*o-iodohippuric*	*73*	*74*			*88*	*73*
5	Tyropanoic	25	8		3	81	32	15	Metrizoic	59	74			99	114
6	Bunamiodyl	39	10		2	53	34	16	Iodopyracet (Na)	101	75		43	72	59
7	Phenobutiodil	81				72									
8	Iobenzamic	22	14			19	44								
9	Iodoalphionic	41	9			66	34								
10	Ph 761/3 B	47	8	25		80	27								

[1] dicarboxylic
[2] MP 271 (Hoey et al 1971)
[3] Tetraiodophthalic acid morpholide (Skagius and Ekemark 1962)
[4] 2 06 and 20 6 mM
1 51 and 15 1 mM
0 2 and 2 0 mM

the control bottle happened to be at the lower end of the range If a sufficiently large number of experiments is run as in the present paper even these margins for random effects may be occasionally exceeded

A great many compounds were kindly donated by the research departments of pharmaceutical houses either directly or through their Swedish distributors The following list facilitates the identification of sources

Table I 1 2 9 12 Schering W Berlin 3 Mallinckrodt St Louis Mo USA 4 13 Leo Halsingborg Sweden 5 Sterling Winthrop Rensselaer N Y USA 6 Guerbet Aulnay sous Bois France 7 Bayer Leverkusen Germany 8 Österreichische Stickstoffwerke Linz Austria 10 Pharmacia, Uppsala Sweden 11 16 Astra, Sodertalje Sweden 15 Nyegaard & Co Oslo Norway

Table II 3 23—25 Astra see I 4 Merck Sharp & Dohme West Point Pa USA 7 Hynson Westcott & Dunning Baltimore Md USA 16 Ciba Basel Switzerland

Table III 2—4 7 9—13 18—20 23 24 27 46 61 70 Astra see I 21 Ferrosan Malmo Sweden 37 Upjohn Kalamazoo USA 35 ICI Macclesfield England 38 62 Lilly Indianapolis Ind USA 40 Winth op Lab New York N Y USA 41 44 Parke Davis & Company Inc Detroit Mich USA 42 48 49 51 53—55 57—59 Smith & Nephew Harlow Essex England 43 Specia, Paris France 45 UPSA Rueil Malmaison France 50 60 68 Merck, Sharp & Dohme see II 52 64 Geigy Basel Switzerland 63 65 Pharmacia see I 66 Leiras Turku Finland 67 Lovens Balle up Denmark

Table IV 13 15 23 Astra, see I 24 Pharmac a, see I 36 46 Hoechst, Frankfurt/Main Germany 38 Hynson Westcott & Dunning see II

Benziodarone Labaz Brussels Belgium

Results

An attempt has been made to divide the material in a logical manner Therefore certain substances occur in several tables Trivial names according to The Merck Index 8th Ed are used wherever possible For brevity substances often are referred to by their ordinal number in a table I 3 means third substance in Table I

TABLE II. Effect on iodipamide uptake of some organic anions known to be secreted into the bile. (For reasons of space the word "acid" is suppressed in this and the following tables.)

			Uptake percent of controls					
			Kidney		Plexus		Eye	
No	Mol. wt.	Millimolarity	0.1	1.0	0.1	1.0	0.1	1.0
1	236	p-Acetylamino hippuric[1]	87	85			78	110
2	194	p-Aminohippuric	100	106			97	66
3	361	Ampicillin	88	91		76	71	93
4	296	Chlorothiazide	93	80		49	115	74
5	376	Fluorescein	58	31		7	112	60
6	*881*	*Indocyanine green*[2]	*72*	*97*			*115*	*84*
7	634	Phenol 3,6-dibromo phthalein disulfonic	44	12		7	98	17
8	354	Phenol red	91	38			146	47
9	838	Sulfobromophthalein	44	9			30	12
10	488	Glycocholic	69	69		22	105	98
11	538	Taurocholic	71	58		14	71	57
12	454	Methotrexate	92	59		67	99	63
13	692	Eosin Y	15	9		4	44	47
14	1018	Rose Bengal	28	12	7		47	23
15	336	Ponceau SX (C.I. 14700)	50	20		23	61	16
16	297	Hydrochlorothiazide	78	67			76	91
17	697	Congo red	94	66		10	93	69
18	698	Bromcresol green	15	0		1	[illegible]	−4
19	670	Bromphenol blue	21	4			58	17
20	423	Chlorphenol red	76	34			71	93
21	466	Thymol blue	39	12			80	56
22	*466*	*Indigo carmine*		*93*				
23	356	Benzylpenicillin[4]		83				90
24	318	Phenolphthalein	61	50			88	73
25	430	Thymolphthalein	83	61			89	76
26	624	Bromthymol blue	36	11			39	−7

[1] = 0.2 and 2.0 mM
[2] = < 0.1 and < 0.33 mM
[3] = *2.15 mM*
[4] = 0.56 mM
[5] = 0.1 and < 1 mM

The tables show the uptake of iodipamide in per cent of controls, as a rule at two concentrations of inhibitor. Since the two concentrations were run with different control bottles, it sometimes happens that the lower concentration apparently causes a deeper inhibition. Because of the small weights of the choroid plexus pieces and the small specific T/M of the uvea pieces, main reliance is placed on the results with kidney cortex. In many cases the inhibitor appears to cause increased uptake, not depression. *Very often this will be due to fortuitously low uptake in the control bottle.* But there are substances which increase uptake (Barany 1973). At the present these stimulatory effects will be neglected.

All available cholegraphic acids were tested. Table I shows the results. It is immediately apparent that all but two depressed uptake and therefore had appreciable affinity. Phenobutiodil had insufficient solubility to be tested above

0 1 mM and may not really be an exception The real exception is iosefamic acid (MP 271) which never was introduced clinically because ———— it was found that opacification to X rays was inferior to that achieved with iodipamide that the opacification took a longer time to develop and that a greater fraction of the drug was excreted into the urine ———— (Hoey *et al* 1971) It is interesting to note that iosefamic acid bears almost exactly the same relation to iothalamic acid as iodipamide to acetrizoic acid Acetrizoic acid is secreted by the renal tubules while iothalamic acid can be used for measurement of glomerular filtration (Sigman *et al* 1965)

The right hand part of Table I shows a number of urographic acids Their affinities are evidently quite low note the very high concentrations used for the first two entries These were run together against a common control which might explain the identical values for kidney if both acids were completely without effect The third entry however acetrizoic acid was run at two separate occasions different from those above and the agreement is a coincidence

The difference between the two series of contrast agents is interesting it seems that natural selection among possible cholegraphic agents has produced compounds with high affinity for the liver like system while the opposite holds for urographic agents In the case of iosefamic acid we have seen natural selection at work

Table II lists 23 other acidic compounds for which Schanker (1968) states that there is at least some evidence for secretion into the bile The compounds are listed in the order used by Schanker and the table contains all the 23 compounds tested which are also listed by Schanker He does not list compounds 24—26 No less than 16 of the 23 depress the L-system Some of them however like PAH and benzyl penicillin have very little effect One would therefore predict that their excretion into the *bile* occurs by a different system than that used for the majority of the listed substances Anyhow the results show a relation between the L system of the kidney eye and choroid plexus and the liver

Table II contains several members of a group which is well known for its affinity to transport mechanisms in the kidney liver eye and brain namely the *fluoresceins phthaleins* and *sulfonphthaleins* Compounds of this group often have very considerable affinity to the L system It is interesting to note the similarity in affinity between the liver function test substance BSP (9) which has to be conjugated before secretion into the bile and its close relative DBSP (7) which is secreted unchanged (Javitt 1964) These two dyes are disulfonic acids and thus carry at least two negative charges However they change their colour in the physiological range and may be partly trivalent Charge of the dye ions seems to be of some but not critical importance dyes with their indicator range on the acid side of physiological pH (*e g* 18 19) are somewhat better inhibitors than those which change their colour on the alkaline side (21 24)

The uncertainty of the plexus and eye values can generate spurious differences between kidney cortex and these tissues Real differences do exist however Congo

TABLE IX. Effect of polyvalent anions on iodipamide uptake

No.	Mol. wt.	Millimolarity	Kidney 0.1	Kidney 1.0	Plexus 0.1	Plexus 1.0	Eye 0.1	Eye 1.0
			Uptake percent of controls					
1	104	Malonic		101				138
2	116	Fumaric		88				89
3	116	Maleic		83				87
4	118	Succinic		116				53
5	132	Glutaric		110				174
6	146	α-Ketoglutaric	100	117		89	155	133
7	146	Adipic		75				171
8	160	Pimelic		88				106
9	166	Phthalic	93			87	120	136
10	166	Isophthalic	87	62			97	77
11	166	Terephtalic	89	39		35	89	60
12	167	Pyridine-2,3-dicarboxylic	88	93				111
13	167	Pyridine-2,5-dicarboxylic	85	92				85
14	167	Pyridine-2,6-dicarboxylic	106	95			151	151
15	167	Pyridine-3,5-dicarboxylic	91	89			97	97
16	175	Diethylsuccinic (high m.p.)	97	123			80	120
17	175	Diethylsuccinic (low m.p.)	102	118			103	114
18	175	Guanidinosuccinic	94	94				76
19	208	Benzylsuccinic	86			173	89	88
20	214	Cyclohexylsuccinic	111	94			95	114
21	261	Naphthalene-2,3-dicarboxylic	83	67			58	79
22	261	Naphthalene-2,6-dicarboxylic	66	54		75	70	73
23	424	Carbenicillin		93				71
24	670	Tetraiodophthalic	114	59			70	48
25	818	α,α-Di(3,5-di-iodo-4-pyridone-N)-pimelic	25	12			72	46
26	192	Citric	90	148		69	104	150
27	238	o-Benzene-disulfonic	86	105			87	40
28	280	2,4-Benzaldehyde-disulfonic	120	113			94	103
29	300	1,5-Naphthalenedisulfonic	108	107			66	108
30	300	2,7-Naphthalenedisulfonic	103	96			50	63
31	311	Tartrazine (C.I. 19140 Food yellow 4)	81	69			61	46
32	336	Ponceau SX (C.I. 14700 Food red 1)	50	20		23	61	16
33	336	Scarlet GN (C.I. 14815)	30	3			26	23
34	412	1,5-Anthraquinonedisulfonic	78	47		65	63	74
35	412	2,6-Anthraquinonedisulfonic	88	58		17	71	59
36	438	3,5-Dihydroxypyrene-8,10-disulfonic	138	43		24	46	37
37	466	Indigocarmine (C.I. 73015)		93				
38	634	Phenol-3,6-dibromophthaleine-disulfonic	44	12		7	98	17
39	697	Congo red (C.I. 22120)	94	66		10	93	69
40	698	Congo corinth G (C.I. 22145)[a]	86	80			81	197
41	725	Benzopurpurine 4 B (C.I. 23500)	82	73			86	205
42	727	Azo blue (C.I. 23680)	99	93			103	141
43	838	Sulfobromophthalein	44	9			30	12
44	881	Indocyanine green	72	92			115	81
45	434	1,3,6-Naphtalene-tri-sulfonic	82	96		110	106	85
46	524	3-Hydroxypyrene-5,8,10-tri-sulfonic	116	74		36	86	46
47	292	EDTA (Na Ca)	94	105			70	114

[a] = 0.47 mM
[b] = 1 and 10 mM
= 2.15 mM
[d] = < 0.1 and < 1 mM
[e] = < 0.1 and < 0.33 mM

TABLE V Effect on iodipamide uptake of detoxication products and analogues

No	Mol wt	Millimolarity	Uptake percent of controls					
			Kidney		Plexus		Eye	
			0 1	1 0	0 1	1 0	0 1	1 0
1	180	Nicotinuric	99	85				12?
2	305	o-Iodohippuric	73	74			88	73
3	194	p-Aminohippuric	100	106			92	66
4	236	p-Acetylaminohippuric[1]	87	85			78	110
5	236	m Acetylaminohippuric	79	72			1?8	83
6	29?	p-Caproylaminohippuric	86	65		59	101	94
7	292	m Caproylaminohippuric[1]	74	47			1?9	7[illegible]
8	228	1 Naphthuric	83	134			109	124
9	228	2 Naphthuric	77	28			50	56
10	274	5 Nitro-1 naphthuric	103				101	
11	244	5 Amino-1 naphthuric	118				124	
12	269	Diphenylacetylglycine	72	34			79	58
13	280	Isophthalyldiglycine	79	105			129	109
14	?80	Terephthalyldiglycine	87	75			119	104
15	488	Glycocholic	69	69		22	105	98
16	449	Glycodesoxycholic	78	27		−1	48	79
17	165	p-Formylaminobenzoic	9?	95		71	129	140
18	179	m Acetylaminobenzoic[1]	98	87			97	165
19	235	p-Caproylaminobenzoic	73	53		110	105	55
20	235	m Caproylaminobenzoic	95	49			133	91
21	230	5 Acetylamino-1 napthoic	113				95	
??	538	Taurocholic	71	58		14	71	52
23	368	Menthylglucuronide	107			41	96	145

[1] = 0 2 and 2 mM

Evidently this other system is not the L system the substance has little affinity

A number of anti inflammatory acid were very effective 41 43 (Soripal® Special) 44 45 (Niflunl® UPSA) and 52 Substance 45 is 2 (m trifluoromethylanilino)nicotinic acid an analogue of flufenamic acid with a pyridine instead of a benzene ring These drugs are all in use or under clinical trial The series of compounds 1A 1T to 16A and 16T are also members of a series screened for analgetic and anti inflammatory activity (Drain *et al* 1971) The A series has a carboxyl group the T series an (acidic) tetrazole ring instead In compounds 14 15 and 16 the NHCO bond is exchanged against a CONH bond The substituents on the rings are always the same in compounds with the same number they are tabulated in Drain *et al* (1971) but not here

The tetrazoles which are about as acid as the A series also were potent uptake inhibitors There was no correlation between the effect on rat foot oedema (reported by Drain *et al* 1971) and uptake inhibition but there seems to be a correlation between the reported oral efficiency in the prevention of phenyl benzoquinone writhing in mice and the L system affinity tested here This matter needs further study

Nalidixic acid (40) was included because of intracranial hypertension following

its use in rare cases (Borcus and Sundstrom 1967) There was no clearcut affinity Azidobenzylpenicillin (61) had activity which is surprising in view of the poor affinity of ampicillin (II 3) Whether or not there is a real difference between Cephalothin (62) and benzylpenicillin (II 23) is doubtful Fusidic acid (67) has considerable affinity which is interesting because of its close similarity to bile acids which are inhibitory (63 65) Novobiocine (68) is known to be excreted by the liver, which fits its inhibitory potency

Several drugs affecting renal handling of uric acid (see Gutman 1966) were tested since it seemed possible that the L-system had something to do with urate Pyrazinoic acid (9) (Yu *et al* 1957) salicylic (16) and m hydroxybenzoic (17) (May and Weiner 1971) did not reach the critical molecular size and had little if any affinity Probenecid (46) phenylbutazone (52) and sulfinpyrazone (64) all had much affinity while carinamide (47) had little On the whole it seems doubtful that the L system is involved in urate transport

The three large anions 32 38 and 39 cause myotonia by inhibition of chloride transport in mammalian muscle fibres (Bryant and Morales Aguilera 1971) At least substance 38 and 39 have affinity to the L system probably also 32

Some aminonaphthalene sulfonic acids (71—74) seem to have more affinity than others, whether this really is so needs further investigation They are weak inhibitors but not weaker than some carboxylic acids in the same weight range

A number of plant growth regulators and analogues (14 28 29 30 33 36) were tested There was affinity in some but never very high The molecular weights are borderline Because of the interest in mammalian toxicity the experiment should be extended to halogenated derivatives

The experiments with flavaspidic acid (66) have been repeated many times and in several species with similar results This compound an anthelminthic causes un conjugated hyperbilirubinaemia in man by inhibition of the uptake of bilirubin into the liver (Nosslin 1963, Nosslin and Morgan 1965) It is quite in keeping with the liver like nature of the L system that it is strongly inhibited by flavaspidic acid

Ethacrynic acid (50) has affinity to the L system but also to the H system (Barany 1973) The almost totally dissociated carbonic anhydrase inhibitor CL 11 366 was studied because of the marked discrepancy between its *in vivo* and *in vitro* potency (Wistrand Rawls and Maren 1960) It has affinity to the L system a fact which indicates that its distribution is affected by at least one transport system

Table IV shows results with polyvalent carboxylic and sulfonic acids

Straight chain dicarboxylic acids (1—8) and substituted succinic acids (16—20) had little effect Among the phthalic acids terephthalic (10) was stronger than isophthalic (11) They were run simultaneously and the experiment repeated (tetra iodophthalic (24) was not superior) The results with the naphthalene dicarboxylic acids (21 22) are analogous the isomer with the larger distance between the carboxyls was stronger Compound 25 is a dimer of iodopyracet prepared by Miss G Stallberg in our laboratory and a strong inhibitor in contrast to iodopyracet Table I

The three disulfonic azo dyes 31 32 and 33 were selected from the series of food dyes investigated for biliary excretion in the rat by Ryan and Wright (1961) This paper also shows the structures Scarlet GN (33) was not found in the bile at all while its isomer Ponceau SX (32) was excreted unchanged to 48 per cent In our experiments Scarlet GN was one of the most potent inhibitors Ponceau SX while still very potent was distinctly less so Tartrazine is a disulfonic monocarboxylic acid It has affinity to the L-system but Ryan and Wright found very little in the bile Such discrepancies are to be expected ability to compete for uptake is only one requirement for secretion and it is also possible that Scarlet GN was changed beyond recognition during its passage through the liver

It is interesting to note that three dyes which all are accepted food dyes at least in some countries all have affinity to the L-system and thus presumably are kept out of the brain and the eye at least partly by that system. On the other hand indigo-carmine (37) which is also a popular food dye has very little affinity to the L-system There remains of course, also the H system which was not tested in the present experiments

The disproportionately deep depression of choroid plexus by Congo red (39) has already been mentioned It has been confirmed in several other rabbit experiments with 3 different batches of medicinal quality Congo red As always in these experiments the pieces of plexus were in the same bottle as pieces of kidney cortex and anterior uvea Thus the reduction of the effective concentration of the dye by the formation of aggregates (which could be quite considerable under our conditions see Quensel 1935) can not explain the discrepancy After the peculiarity of Congo red had been discovered the three others dyes 40—42, very closely related to Congo red were tested These were for histology and not of medicinal quality They did not dissolve well in our medium and were weak inhibitors Maybe they all form larger aggregates than Congo red under our conditions it is known (see Robinson and Mills 1931) that Benzopurpurin 4 B (41) flocculates at about 100 mM NaCl while Congo red tolerates almost 10 times as much

We were also unable properly to dissolve indocyanine green (44) in our buffer This dye definitely is excreted by the liver in several species (see Smith 1970) and it would be interesting if it really had no affinity to the L-system

EDTA (Na Ca) was tested since it has been claimed to be secreted by a separate anion system (Heller and Vostal 1964) Evidently this it not the L-system

An overview of the polyvalent unconjugated acids shows that in this case too molecular weight plays a role for affinity It is possible that the borderline mol wt is somewhat higher in the polyvalent case but since very few non sulfonic acids with mol wt above 300 were tested this is not certain

Table V shows results with a few detoxication products and analogs of such compounds There were 7 glycine conjugates which have unconjugated counterparts in Table II—IV Despite the fact that glycine conjugation increases the mol wt the unconjugated *acids were stronger* inhibitors than the conjugated ones even if the difference sometimes was insignificantly small as between cholic and glycocholic. It

is interesting that not only 2 naphthoic acid (III 26) was a stronger inhibitor than 1 naphthoic (III 25) but that the same relation was obtained with the naphthuric acids (V 8, 9)

Finally a few findings with odd substances Phlorizin which is excreted by the liver in the dog (Jenner and Smyth 1957) and the chicken (Sperber 1963) at 1 mM resulted in 81 7 (!) and 120 percent uptake in kidney plexus and eye respectively Thus *it is somewhat similar to* Congo red in depressing choroid plexus disproportionately

Pilocarpine which inhibits the uptake of iodopyracet in uvea (Walinder 1966) and of o iodohippurate in kidney cortex (Barany 1970) to some extent had no inhibitory effect on the L system

Benziodarone was tested because it inhibits biliary excretion of the synthetic glutathione conjugate of BSP but not the excretion of unconjugated BSP or DBSP (Priestly and Plaa 1969) At 0 5 mM, uptake in kidney was 76 and in uvea 50 percent a borderline depression which should be investigated further

A very long acting sulfonamide drug sulfalene was tested at 1 mM because of the possibility that *it* undergoes active reabsorption in the kidney The figures obtained for kidney plexus and uvea were 87 66 and 91 none of them significant

Discussion

The data even if they are only results of rough screening experiments provide further support for the notion that the L system is liver like Cholegraphic agents which have been selected by experience as liver excreted had affinity urographic agents little or none Among a group of liver secreted substances the majority had affinity to the L system The known molecular size effect on biliary excretion was clearly visible also on the affinity to the L system Flavaspidic acid a known inhibitor of biliary transport of bilirubin inhibits the L system too

No specific structural requirement for the absence or presence of affinity to the L system has been found Acidic substances of very different structure different valency and different pKa had affinity others sometimes surprisingly none

There is some evidence that the L system is composite and that one component is even less sensitive to hippurate than the other (Barany 1972) In the reported data hippurate concentration varied from 8 9 to 30 mM Therefore *it is possible that* components of the L system were differently affected even in the control slices If for no other reason small differences in the listed degrees of uptake should be viewed with suspicion

If an inhibitor had an appreciable effect on uptake in kidney cortex there usually was an effect also on plexus and uvea and *vice versa* even if the scatter was great Some curious discrepancies such as Congo red and phlorizin exist however and can not as yet be explained It is possible that the components of *the L-system* occur in different proportions in different tissues

The results indirectly shed some light on organic acid excretory systems of the

liver Not all liver excreted acids have appreciable affinity to the L system This indicates that the liver like the kidney has several such systems some of which do not overlap with the L system of the kidney A multiplicity of liver anion transport system has already been postulated by Alpert *et al* (1969) on the basis of studies in mutant sheep Taurocholic acid and BSP seem to use partly different systems This subdivision probably does not correspond to the one just mentioned

The fact that the L-system and the liver excretory system both are sensitive to molecular size is significant The size requirements for biliary excretion have been established by studies of the total excretory process This involves many steps maybe even reabsorption (Clark *et al* 1971) several of which could be size sensitive In the L-system size sensitivity is apparent already at the stage where substances compete with iodipamide for uptake or possibly intracellular binding (*cf* Barany 1972 1973) If this also holds for the liver which is probable then size would be an early selection factor for biliary excretion not a late one as suggested by Clark *et al* (1971)

Quite a number of drugs have affinity to the L system in the kidney cortex and other extrahepatic tissues This not only indicates something about another probable route of excretion (the liver) but also implies that these drugs are not passively distributed between the blood stream and the eye or brain Thus the L system very probably fulfils the same function from a pharmacokinetic point of view for liver excreted drugs as the H system for drugs mainly excreted by the kidney it affects the *in vivo* distribution and offers opportunities for drug interactions

Most substances of Table V were synthetized by Miss Gunnel Stallberg and many commercial ones in Table II –IV recrystallized by Mr Folke Berglund
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Recurrent Inhibition from Motor Axon Collaterals of Ventral Spinocerebellar Tract Neurones

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Abstract

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Recurrent inhibition from motor axon collaterals was searched for in intracellularly recorded ventral spinocerebellar tract (VSCT) neurones in the cat A small proportion of the investigated cells received inhibition on antidromic stimulation of ventral roots The IPSP was evoked by α fibres had a segmental latency indicating a disynaptic linkage from motor axon collaterals and a duration of about 50 ms. The inhibited cells received monosynaptic excitation from group Ia muscle spindle afferents The inhibition could be demonstrated also on the VSCT mass discharge evoked by stimulation of Ia afferents The ventral roots giving rise to the inhibition in a given VSCT cell were the same as the ventral roots which give recurrent inhibition in α motoneurones and Ia inhibitory interneurones excited by Ia afferents of the same muscle as the VSCT cell The results indicate that Renshaw cells terminating on α motoneurones and Ia inhibitory interneurones also send collaterals to some VSCT neurones This finding strongly supports the idea that the VSCT relays information about the transmission in interneuronal inhibitory reflex pathways to motoneurones (Lundberg 1971)

The ventral spinocerebellar tract (VSCT) neurones receive a very complex synaptic input from different primary afferents and descending tracts (Oscarsson 1957 Magni and Oscarsson 1961 Lundberg and Weight 1971 Baldissera and Bruggencate 1969 Baldissera and Weight 1969) Lundberg (1971) has related these effects to the known convergence on interneurones in different reflex pathways to motoneurones and has forwarded the hypothesis that VSCT neurones signal information about the transmission in interneuronal inhibitory reflex pathways to motoneurones Such information might be needed for cerebellum due to the extensive convergence onto the interneurones in these pathways from different primary afferents and descending tracts (Lundberg 1969 1970 Hultborn 1972 a b) According to the hypothesis the VCST relays information about each specific pathway in three different ways One group of VSCT cells may through collateral connexions receive both the excitatory input and the inhibitory output from the last order interneurone

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in a pathway Such cells may be considered as input output comparators Another group of VSCT cells may receive only the synaptic effects converging onto this interneurone or a fraction of these effects These cells may in a wider sense contribute to the comparator function of the VSCT by providing a reference for the input output comparing cells The third group of VSCT cells may compare directly through collateral connexions the effects from excitatory and inhibitory pathways converging onto α motoneurones This may provide cerebellum with information regarding how effectively the inhibition counteracts the excitation at the output stage of the spinal cord

The hypothesis has been discussed in most detail in relation to the well known inhibitory pathway from large muscle spindle (Ia) afferents to motoneurones The interneurones in this pathway receive synaptic effects, not only from primary afferents and descending tracts but also from the efferent side—namely recurrent inhibition through motor axon collaterals and Renshaw cells (Hultborn *et al* 1971 *a b*) Since the hypothesis assumes collateral connexions to some VSCT neurones from such Ia inhibitory interneurones it has gained support from the demonstration that Ia IPSPs in VSCT neurones can be depressed by antidromic stimulation of motor axons (Gustafsson and Lindstrom 1970 1973) This finding indicates that the same Ia inhibitory interneurones terminate on α motoneurones and VSCT cells

The present study provides further evidence that the convergence onto VSCT neurones corresponds to the excitatory and inhibitory convergence on last order inhibitory interneurones or α motoneurones Since Ia inhibitory interneurones and α motoneurones receive recurrent inhibition from motor axon collaterals one would expect from Lundberg s hypothesis to find some Ia excited VSCT neurones with such recurrent inhibition The results demonstrate that VSCT neurones with this convergence do indeed exist

Methods

Intracellular recordings The experiments were performed on adult cats operated under ether and subsequently anaesthetized with alpha chloralose (initial dose 50—60 mg/kg i v usually additional 10—20 mg/kg after 5—8 h) The animals were immobilized which gallamine triethiodide (Flaxedil May and Baker Ltd) and artificially respired Blood pressure and expired CO_2 were monitored throughout the experiments Rectal temperature was kept within 37—39 C

The spinal cord was exposed by laminectomy of the thoracic vertebrae Th 11 and Th 12 and the lumbar vertebrae L4 —L6 In the low thoracic region the dorsal column and the left spinal half (ipsilateral to the recording site) were removed for about 2 cm On the same side the ventral roots L5—S1 were transected and their proximal ends were mounted on bipolar stimulating electrodes Several cutaneous and muscle nerves in the left hindlimb were also dissected (see Hultborn *et al* 1971 *a*) and mounted on bipolar stimulating electrodes A bipolar electrode was used to stimulate the surface of the right thoracic hemicord Rectangular pulses of 0 1 ms duration were used in all cases In about half of the experiments the ipsilateral (left) anterior lobe of cerebellum was exposed by posterior occipital lobectomy and partial removal of the bony tentorium The cerebellar surface was stimulated with rectangular pulses (0 2 ms duration) through a spring mounted movable silver ball electrode (cathode) the current return electrode being a large silver wire burried in the neck muscles The skin flaps around the exposed areas of the spinal cord and the hindlimb were sewn up to form pools filled with paraffin oil while the exposed cerebellar surface was kept moistened with warm saline

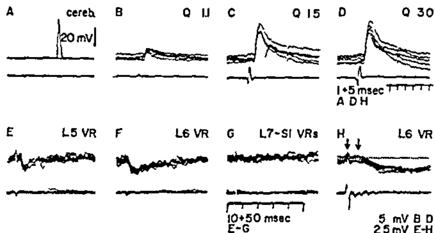


Fig 1 Inhibition of a VSCT neurone by antidromic stimulation of ventral roots (VRs) Upper traces in each pair show intracellular responses lower traces nerve volleys recorded triphasically from the dorsal root entry zone Positivity is indicated upwards in the intracellular recordings and downwards in nerve volley recordings A antidromic spike evoked by stimulation of the ipsilateral anterior lobe of the cerebellum (cereb) B—D monosynaptic EPSPs evoked by Ia afferents from the nerve to quadriceps femoris (Q) The strength of the nerve stimulation in multiples of the threshold strength is indicated above the records In E—G are shown the effect of antidromic stimulation of the ventral roots (VRs) L5 L6 and L7—S1 respectively In this and following figures the strength of stimulation of the VRs is supramaximal for *a* fibres The IPSP from the VR L6 is shown with higher sweep peed in H The superimposed dotted line indicates the extracellular field potential recorded just outside the cell The interval between the arrows represent the central latency of the IPSP Voltage calibrations are for intracellular responses

Intracellular recording was made from cell bodies of the VSCT on the left side in the lumbar segments L4—L5 The cells were identified as belonging to the VSCT by antidromic invasion on stimulation of either the contralateral (right) spinal half at low thoracic level (Burke *et al* 1971) or the ipsilateral (left) anterior lobe of cerebellum (Burke *et al* 1971) The cells were considered to have a cerebellar termination f the threshold strength for antidromic activation was 2 mA or less Glass micropipette electrodes were used and they were f lled with 2 M K citrate had broken tips with a diameter of 1 5—2 0 *u*m and resistances of 3—6 MΩ Conventional techniques of amplificat on and recording were used The cathode follower has been described by Eide (1968) Orthodromic and antidromic nerve volleys were recorded from the surface of the spinal cord between a s l er ball electrode placed clo e to the dorsal root entry zone in L6 and an indifferent electrode in the back muscles Differentiation between afferent f bre groups we e done as desc bed by Bradley and Eccles (1953) Eccles *et al* (1957) and R M Eccles and Lundb rg (1959) The stimulus repetition rate was 0 5—1 Hz

Mass discharge reco d ngs These experiments were performed on precoll cularly decerebrated cats to allow the use of subanesthetic doses of pentobarbiton sodium (Nembutal Abbott see Results) The VSCT mass discharge was recorded from a small dissected fascicle of the right lateral funicle at low tho acic le el Usually only the dorsal columns were emoved at this level the left spinal half a d the rest of the right half being left intact to have the be t po sible suppression of polysynaptic discharges in the dissected fascicle (Holmqvist *et al* 1960) To disclose even the smallest VSCT discharge the recordings were averaged with a Hewlett Packard averaging computer Usually 128 test responses and 128 responses conditioned by a preceding maximal stimulation of the entral root L5—S1 were alternated to two halves of the computer memory In other respects the experimental arrangements and the na ntainence of the preparation were as decribed abov

Abbreviat ons The following abbreviations are used VSCT ventral sp nocerebellar tract EPSP excitatory postsynaptic potential IPSP inhib tory po tsynaptic potential VR ventral root PBSt posterior biceps and semitendinosus Q quadric ps femoris

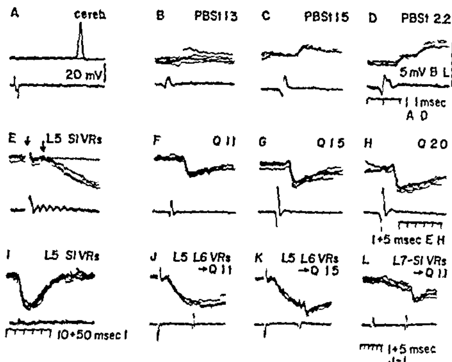


Fig 2 Intracellular recordings from another VSCT cell inhibited by ventral root stimulation A antidromic spike evoked from cerebellum, E and I recurrent IISI evoked by stimulation of VRs L5—S1 and shown with two different sweep speeds The extracellular field potential is indicated in E as in Fig 1 B—D monosynaptic EISPs evoked from Ia afferents in the nerve to posterior biceps-semitendinosus (PBSt) The late EPSP in D is presumably evoked disynaptically from Ib afferents F—H disynaptic IISIs from Ia afferents in the Q nerve J and L show the IPSP in F conditioned by a preceding stimulation of the VRs L5—L6 and L7—S1 respectively K shows the larger IISP of G conditioned by a volley in the VRs L5—L6 as in J Further details as in Fig 1

Results

Intracellular recordings Altogether five out of more than 200 intracellularly recorded VSCT neurones were inhibited by antidromic stimulation of ventral roots These cells were encountered in four different cats Three of the cells were identified as belonging to the VSCT by antidromic activation from the anterior lobe of cerebellum ipsilateral to the recording side (*cf* Methods) whereas the other two were encountered in experiments on spinal cats and therefore identified by antidromic activation from the contralateral (right) spinal half at low thoracic level (*cf* Burke *et al* 1971) All five were found in the L5 segment and they were located more medially in the spinal cord than most other VSCT neurones (Jankowska Lindstrom and Schomburg to be published)

Records from two of the cells antidromically identified from cerebellum are shown in Fig 1 and 2 The cell in Fig 1 received inhibition from the VRs L5 (E) and L6 (F) but not from the VRs L7—S1 (G) while the cell in Fig 2 could be

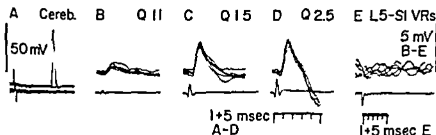


Fig 3 Intracellular recordings from a VSCT neurone without inhibition from ventral roots A, antidromic spike evoked from cerebellum B—D monosynaptic EPSPs evoked by Ia afferents in the Q nerve E lack of effect of maximal stimulation of VRs L5—S1 Further details as in Fig 1

inhibited from the VRs L5—S1 stimulated separately (not illustrated) The IPSPs appeared with the α volley and were maximal at a stimulation strength of about 1 5 times threshold for α fibres The minimal central latencies varied between 1 4 and 2 3 ms (1 4 ms for the cell in Fig 1 and 1 6 ms for the cell in Fig 2) The latencies were measured from the first positive peak of the efferent volleys to the point where the intracellularly recorded potentials deviated from the superimposed field potentials recorded just outside the cells (*cf* arrows in Fig 1 H and Fig 2 E) The latencies are within the ranges found for recurrent inhibition of α motoneurones (Eccles *et al* 1954) and Ia inhibitory interneurones (Hultborn *et al* 1971 a b) and indicate that also the recurrent pathway to the VSCT cells is disynaptic The time to peak of the IPSPs was about 8 ms and the duration about 50 ms Thus the time course of these IPSPs corresponds to the time course of recurrent IPSPs in α motoneurones and Ia inhibitory interneurones (Eccles *et al* 1954 Hultborn *et al* 1971 a b)

The VSCT cells with inhibition from ventral roots also received monosynaptic excitation from muscle spindle Ia afferents In four cells the excitation was supplied by Ia afferents from the nerve to the knee extensor Q while one cell was excited from the nerve to the knee flexors PBSt These two types of Ia convergences are illustrated in Fig 1 and 2 The monosynaptic EPSPs grew with the low threshold component of the group I volley representing the faster conducting Ia fibres (B—C) and did not increase further in amplitude when the stimulus strength wa increased to activate also the slower conducting Ib fibres (Fig 1 and 2 D Bradley and Eccles 1953 Eccles *et al* 1957) *All cells excited from the Q nerve (cf Fig 1)* received inhibition only from the VRs L5 (E) and L6 (F) but not from the VRs L7—S1 (G) This is similar to most Q excited α motoneurones and Ia inhibitory interneurones (Hultborn *et al* 1971 a)

It should be stressed that the VSCT cells with inhibition from ventral roots constitute only a small fraction of the total sample of tested VSCT cells with monosynaptic excitation from muscle spindle Ia afferents (5 out of about 60 cells) An example of a VSCT cell without inhibition from the ventral roots is shown in Fig 3 The Ia excitation was supplied by the Q nerve (B—D) as for the cell in Fig 1

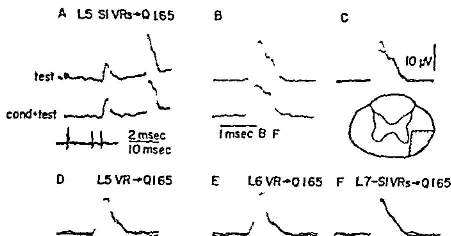


Fig. 4. Recurrent effects on VSCT mass discharge recorded monophasically from a small dissected fascicle of the right lateral funicle of the spinal cord at the level of Th 11—Th 12. The approximate extension of the fascicle is indicated by the hatched area of the inserted diagram in C. Upper trace in A shows the test responses evoked by a double stimulation of the Q nerve at a strength maximal for Ia fibres, middle trace shows this test preceding by a conditioning stimulation of the VRs L5—S1. The records were obtained by averaging 128 unconditioned and 128 conditioned responses alternated to two halves of the memory of an averaging computer. The conditioning testing interval is seen from the recorded incoming nerve volleys lowermost in A, taken with a slower sweep speed. The time calibration bar in A is 10 ms for the lowermost trace and 2 msec for the upper traces. The second VSCT discharges are shown expanded in B and superimposed in C. D—F show the unconditioned and conditioned second responses expanded and superimposed as in C but with the conditioning volley evoked in the VRs L5, L6 and L7—S1 respectively. Further details in the text.

However, simultaneous stimulation of the VRs L5—S1 supramaximal for α fibres was without any effect (E). Of the other VSCT cells without inhibition from the ventral roots about 60 cells received monosynaptic excitation from Ib afferents (cf. Eccles et al. 1961) while the majority of the remainder received no monosynaptic excitation from any of the dissected nerves.

Only one of the cells with inhibition from VRs received inhibition also from Ia afferents, namely the cell of Fig. 2. The IPSP was evoked disynaptically from the lowest threshold afferents from the antagonist Q (F—H). Fig. 2 J shows that this IPSP, evoked at the same stimulation strength as in F, was virtually abolished by a preceding antidromic volley in the VRs L5—L6, while the VRs L7—S1 had a negligible effect (L). The decrease of the IPSP on conditioning stimulation of the VRs L5—L6 can only partly be due to the hyperpolarization and the conductance increase during the recurrent IPSP, since a similar conditioning volley had proportionally much smaller effect on the Ia IPSP evoked at higher strength of stimulation of the Q nerve (cf. F—J and G—K). It can thus be concluded that this Ia IPSP was evoked through Ia inhibitory interneurones susceptible to recurrent inhibition from motor axon collaterals similarly to those mediating Ia reciprocal inhibition to motoneurones (Hultborn et al. 1971 a, b, cf. also Hultborn 1972 b).

All cells with inhibition from the ventral roots also received polysynaptic inhibition and sometimes also polysynaptic excitation from high threshold muscle afferents and cutaneous afferents like other VSCT neurones (Oscarsson 1957 Lundberg and Weight 1971) The cell in Fig 2 in addition received a late EPSP from the PBSt nerve (D) presumably evoked disynaptically from Ib afferents

Mass discharge recordings Although many VSCT neurones receive monosynaptic excitation from Ia afferents (Lundberg and Weight 1971) no VSCT mass discharge has earlier been found on selective stimulation of Ia afferents (Oscarsson 1956 Lundberg and Weight 1971) However small VSCT mass discharges evoked by Ia volleys were disclosed in several cats by averaging Fig 4 illustrates that inhibition by ventral root stimulation could be demonstrated also on such VSCT responses These records were obtained from a dissected ventrolateral fascicle on the contralateral (right) side of the spinal cord at the level of Th 11—Th 12 (see inserted diagram in Fig 4C) The cat was precollicularly decerebrated to have the best possible suppression of polysynaptic discharges in the fascicle (*cf* Holmqvist *et al* 1960) In unanesthetized animals recurrent facilitation of VSCT neurones from motor axon collaterals can easily be demonstrated on the VSCT mass discharge (Gustafsson and Lindstrom 1973 *cf* Hultborn *et al* 1971 d) This facilitation would effectively mask any recurrent inhibition of some VSCT neurones Intermittent small doses of Nembutal were therefore supplied intravenously to abolish this recurrent facilitation (Hultborn *et al* 1971 d) The level of anesthesia was very critical and had to be balanced so that the VSCT discharge at the same time was not too much depressed The upper trace in A shows VSCT mass discharges evoked by double stimulation of the Q nerve at a strength maximal for Ia afferents Double stimuli were used to increase the number of fired VSCT neurones The middle trace in A shows the same responses conditioned by a preceding maximal stimulation of the VRs L5—S1 To have better time resolution of the mass discharges only the part of the sweep containing these responses was averaged The conditioning testing interval is indicated by the recorded incoming nerve volleys lowermost in A In this case only the second test discharge was susceptible to recurrent inhibition (see below) The depression is best seen from the expanded and superimposed records in B and C As shown by the similarly superimposed records in D—F the VSCT discharge evoked from the Q nerve was depressed both from the VRs L5 and L6 while the VRs L7—S1 were without significant effect The ventral root stimulation alone did not give rise to any ascending discharge indicating that VSCT cells cannot be fired directly from motor axon collaterals

The effect shown in Fig 4 could not be demonstrated on VSCT discharges evoked by stimulation of the Q nerve at a strength slightly above or below the one used This means that at these strengths no VSCT neurones were fired which could be moved into the subliminal fringe by a conditioning ventral root volley The same phenomenon explains the lack of conditioning effect on the first response in Fig 4 A Similar results have been obtained in two other cats Since other observations indicate that the Ia excited VSCT neurones have a rather large subliminal

fringe (cf the double stimulation in A) these findings must imply that only a small fraction of the Ia excited VSCT neurones receives inhibition from motor axons as indicated already by the microelectrode sampling

Discussion

The inhibition found in some VSCT neurones on stimulation of ventral roots resembled the recurrent inhibition from motor axon collaterals to α motoneurones (Eccles et al 1954) and Ia inhibitory interneurones (Hultborn et al 1971 a b) Thus the IPSPs were evoked from α fibres, had central latencies indicating a disynaptic linkage from motor axons to the VSCT cells and durations in the same order as recurrent IPSPs in those neurones It seems therefore reasonable to assume that the recurrent effect to the VSCT cells was evoked through a pathway identical with the recurrent inhibitory path to α motoneurones and Ia inhibitory interneurones

The results from microelectrode sampling and mass discharge recordings demonstrate that only a small fraction of the VSCT cells receives recurrent inhibition from motor axon collaterals This is in contrast to α motoneurones and Ia inhibitory interneurones the great majority of which receives such an inhibition However it seems unlikely that the recurrent effect to the VSCT cells was due to aberrant" connexions and thus functionally insignificant Firstly the effect was observed in several cats and secondly VSCT cells in general seem to have such a highly differentiated input that one cell hardly resembles another (Lundberg and Weight 1971) On the other hand the infrequent occurrence of this inhibition in VSCT cells suggests that the effect should not be considered in the same functional terms as the recurrent inhibition of α motoneurones and Ia inhibitory interneurones The inhibition rather seems to be a consequence of the specific information forwarded by the VSCT cells which receive this effect It therefore seems highly significant that the VSCT neurones with recurrent inhibition also received monosynaptic excitation from Ia afferents like α motoneurones and Ia inhibitory interneurones This pattern of convergence is of interest in relation to the hypothesis (Lundberg 1971) that VSCT neurones receive through collateral connexions an excitatory and inhibitory convergence similar to last order inhibitory interneurones or α motoneurones (see Introduction)

The present results cannot prove if the recurrent inhibition of the VSCT neurones and e g Ia inhibitory interneurones with the same Ia excitation is evoked through branches of the same Renshaw cells (as required by the hypothesis) and not through separate channels However there are no indications of a functional differentiation of Renshaw cells with respect to the type of their target neurones On the contrary a detailed comparison of the pattern of convergence from different efferent nerves onto α motoneurones and Ia inhibitory interneurones led Hultborn et al (1971 c) to conclude that the same Renshaw cells terminate on α motoneurones and Ia inhibitory interneurones with a similar Ia input The most pronounced recurrent inhibi-

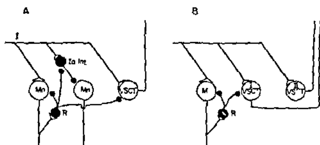


Fig 5 A schematic illustration of collateral connexions from the same Renshaw cells (R) to synergistic α motoneurones (sMn) Ia inhibitory interneurones (Ia Int) and VSCT neurones supplied with monosynaptic excitation from the same Ia afferents Black cells represent inhibitory interneurones aMn antagonistic α motoneurones B as in A but showing collateral connexions from the same Ia afferents to VSCT neurones with and without recurrent inhibition Connexions to Ia inhibitory interneurones omitted in this scheme

tion in both groups of cells is evoked from efferents to the muscles which supplied Ia excitation to the cells while efferents to antagonists are without effect This means that due to the rostrocaudal distribution of the motor nuclei α motoneurones and Ia inhibitory interneurones with excitation from *e g* the quadriceps nerve receive strong recurrent inhibition from the VRs L5 and L6 while the VRs L7 and S1 are mostly without effect (Hultborn *et al* 1971 a c) The present results demonstrate that the same holds true also for VSCT cells excited from the quadriceps nerve It is hard to believe that this parallelism between the convergence of Ia excitation and recurrent inhibition onto the VSCT cells and α motoneurones or Ia inhibitory interneurones is a chance event Accordingly it seems justified to postulate that the recurrent inhibition of the VSCT cells is indeed evoked through collaterals of Renshaw cells which terminate on α motoneurones and Ia inhibitory interneurones with a similar excitatory input from Ia afferents as the VSCT cells This suggested organization is illustrated schematically in Fig 5 A

What type of information is then supplied by this group of VSCT cells? None of them obtained disynaptic inhibition from the same Ia afferents as those supplying monosynaptic excitation as would be required for the input output comparing cells (Lundberg 1971 *cf* Introduction) They seem instead to correspond to VSCT cells with excitatory and inhibitory convergence similar to that of last order interneurones (in this case Ia inhibitory interneurones) or α motoneurones (*cf* Introduction) Since Ia inhibitory interneurones and α motoneurones receive excitation and inhibition from most afferent and descending systems in parallel including reciprocal Ia inhibition (Fig 2 *cf* Hultborn 1972 b) it is not possible to distinguish between these two alternatives from the present results In the following they will therefore be considered as equivalent although functionally they may not be so

Only a small proportion of the Ia excited VSCT cells received recurrent inhibition This indicates that individual VSCT cells obtain only a part of the total input to a specific group of interneurones or motoneurones (Lundberg 1971) The present group of VSCT cells receives Ia excitation plus recurrent inhibition Other VSCT cells may obtain only Ia excitation or Ia excitation together with effects from any of

the descending systems known to affect Ia inhibitory interneurones (or motoneurones) Combined with input-output comparing cells this type of arrangements will supply cerebellum with detailed information not only about the output from the group of interneurones of interest but also about the input to these cells through different channels. This might be required since a large part of the convergent effect onto these interneurones (especially the inhibition) is in turn evoked through interneuronal pathways where the interneurones are under extensive control from different sources It is possible that each combination of convergence is represented by only a few VSCT cells in each animal

To illustrate the above discussion By comparing the output of Ia comparator VSCT cells with the output from the other type of Ia excited VSCT cells with and without recurrent inhibition the cerebellum can determine to which extent the output from the Ia inhibitory interneurones is determined by the Ia excitation and by the recurrent inhibition respectively Seen from another point of view this arrangement provides cerebellum with information about the transmission through the recurrent inhibitory pathway There seems to be no direct information channel for the recurrent pathway through the VSCT since there was no VSCT cells fired by the ventral root stimulation Information about the recurrent pathway might however be needed since the Renshaw cells are under excitatory and inhibitory control from several systems (Haase and van der Meulen 1961 Wilson *et al* 1964, MacLean and Leffman 1967 Bergmans *et al* 1969 Ryall 1970) If the cerebellum compares the output from two similarly Ia excited VSCT cells with and without recurrent inhibition (Fig 5 B) the difference in firing frequency between these two cells may give a measure of the strength of the recurrent inhibition of α motoneurones and Ia inhibitory interneurones

Summarizing the present finding of recurrent inhibition from motor axon collaterals of Ia excited VSCT neurones strongly supports the hypothesis of Lundberg (1971) that the VSCT signals information about the convergent actions onto motoneurones and last order interneurones in segmental inhibitory pathways to motoneurones The occurrence of this effect in only a small fraction of the VSCT cells strengthens the notion that individual VSCT neurones forward a highly specific information

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Incorporation of Guanethidine into Isolated Rat Peritoneal Mast Cells and Mast Cell Granules in Vitro

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Abstract

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The incorporation of guanethidine into isolated rat peritoneal mast cells was studied in vitro The incorporation of the drug is dependent upon 1 temperature 2 the pH of the medium 3 the presence of Ca ions in the medium it 4 shows saturation characteristics and is 5 inhibited by 5 hydroxytryptamine and tricyclic antidepressants Of the metabolic inhibitors tested, only FCCP reduced incorporation of guanethidine The intracellular location of guanethidine in mast cells is mainly in the amine storing organelles It is concluded that the incorporation of guanethidine is partly due to an active transport mechanism located in the cell membrane while the incorporation of the drug into isolated membrane free mast cell granules is a passive process resulting in binding to sites in the granule matrix

Guanethidine causes a long lasting depletion of peripheral stores of Noradrenaline (NA) and makes adrenergic neurones unresponsive to stimulation (for refs see e g Costa 1966) The drug is probably transported into the adrenergic nerves via a mechanism located in the cell membrane (Obianwu Stitzel and Lundborg 1968 Mitchell and Oates 1970 Mitchell *et al* 1970) According to Schanker and Morrison (1965) guanethidine accumulates in rat heart tissue *in vitro* The incorporation is in part due to an active transport mechanism Recently Maitre and Staehelin (1971) reported that the uptake of this drug into NA storage particles of rat heart was reserpine sensitive Besides getting bound to specific NA storage sites guanethidine is also bound to unspecific sites in sympathetically innervated tissue (Chang Costa and Brodie 1965)

Human platelets take up guanethidine by an energy requiring mechanism (Boullin and O Brien 1969) Guanethidine affects the 5 hydroxytryptamine (5 HT) content of rat peritoneal mast cells *in vitro* by lowering the endogenous and depressing the incorporation of exogenous 5 HT into isolated mast cell granules (Jansson 1971) In a preliminary report, the incorporation of guanethidine and reserpine into mast cells was reported to take place by different mechanisms (Gripenberg and Jansson 1971) and later the incorporation of reserpine was shown to be a passive process (Gripen

berg Jansson and Partanen 1972) The results to be presented in this paper indicate that the incorporation of guanethidine into isolated mast cells *in vitro* is partly due to an active transport mechanism while the incorporation into isolated mast cell granules is a passive process

Material and methods

Adult albino rats of both sexes of the Sprague Dawley strain were u d The mixed pleural and peritoneal cells were washed out from the pleural and peritoneal cavities each with 10 ml of incubation medium as described earlier (Jansson 1971)

Isolation of mast cells and mast cell granules
Isolation of mast cells was performed with minor modifications of a method described by U nas and Thon (1959) The yield of cells from 3—4 rats was layered on top of 12 ml of 30 % Ficoll (Pharmacia) in saline supplemented with 0 1 % albumine and glucose A practically pure mast cell fraction was collected from the Ficoll layer after centrifugation at 350 × *g* for 1 min at room temperature The cells were washed free from Ficoll with two changes of fresh medium resuspended and counted in a Buerker chamber prior to incubation experiments

Mast cell granules were isolated as described by Lagunoff *et al* (1964) and by Thon and Uvnas (1966) The cells were washed with 0 32 M sucrose pH 6 9 resuspended in sucrose and degranulated by freezing and thawing Centrifugation of the suspension for 5 min at 350 × *g* produced a pellet consisting of cell debris and granules and further centrifugation of the supernatant for 30 min at 2700 × g produced an almost pure mast cell granule fraction The centrifugations were carried out at room temperature

Incubation technique
The cells were washed out and incubated in a physiological electrolyte solution buffered with tris (hydroxymethyl) amino methane (Sigma 7—9® Sigma Chemical Co) of the following composition NaCl 136 mM KCL 5 6 mM $CaCl_2$ 2 2 mM MgCl 1 3 mM NaH_2HPO_4/ Na HPO_4 1.3 mM Tris HCl buffer 20 0 mM bovine serum albumine and glucose 0 1 % pH 7.35 at 37 C 7 42 at 23 C and 7 52 at 4 C

In experim nts concerning the pH dependence of the incorporation of guanethidine sodium phosphate buffer at an equ molar concentration was used instead of Tris-HCl in the pH range 6 0– 7 ?

The incubations were carried out in 10 ml teflon stoppered tubes in a water bath under agitation at 37 C unless otherwise stated The incubation volume was 1 0 ml with an a erage cell concentration of 0 5 × 10^4 mast cells/ml After incubation the cells were spun down and washed with two changes of fresh medium and with one change of 0 9 NaCl pH 7 1 in order to minimize the blanc readings due to the Tris HCl buffer in the incubation medium. The washed cell pellets were resuspended in 0 5 ml 0 1 N HCl The mast cell granules were incubated in 0 32 M sucrose pH 6 9 at 23 C washed with two changes of fresh sucrose and re suspended in 0 1 N HCl The effect of arious drugs on the incorporation of guanethidine was stud ed by the add tion of drugs 15 min prior to the addition of guanethidine All glass are includi g pipettes was siliconized (Siliclad® Clay Adams Co) The samples were sto ed at −35 C for not more than 3 days prior to the determination of guanethidine and 5 HT

Dete mi ation of guanethidine
Guanethidine was determined spectrophotofluo ometrically acco ding to a semi micro modification of a method described by Schanker and Morrison (1965) The f nal sample volume was 100 µl The fluorescen e was read at 495 nm after activation at 400 nm in an Aminco-Bowman spectrophotofluorometer The linearity of the method was c nfirmed and extended down to a concentration of 0 15 nmol/ml by the present semi mic o modificat on The ecovery of the method was good using biological mater al 97 0 ± 4 4 % (means a d S E) of the theoretical amount was reco ered from internal standards The ariation oefficient of the method was 4 9 % The possible nt rference of biological metabol tes of guanethidine ith the method was not evaluated Guanethidine standards were made up from a stock solution containing 5 µmol guanethidin /ml water None of the drugs u ed inte fe ed with the method at the concent ations u ed The amounts of guaneth d ne reported were corrected for blan alues

Determi at on of 5 HT
The 5 HT content of the cell lar material was determ ned pectrophotofluorometricall using

TABLE I Incorporation of guanethidine into isolated rat peritoneal mast cells as a function of the incubation temperature Incorporation expressed as nmole/10^6 mast cells

0 C	23 C	37 C
0 115 ± 0 072	0 176 ± 0 024	0 505 ± 0 067

Incubation as described in text with guanethidine at 5×10^{-5} M for 60 min under agitation Means and S E of 3 expts one in duplicate two in triplicate

TABLE II Incorporation of guanethidine into isolated mast cells as a function of exogenous concentration *Incorporation as nmol/10 mast cells*

$5 \times 10^{-}$	$2 5 \times 10^{-}$	$5 \times 10^{-}$	10^{-4}	$2 5 \times 10^{-4}$
0 063 ± 0 023	0 242 ± 0 036	0 399 ± 0 059	0 616 ± 0 104	1 15 ± 0 110

Incubation of isolated mast cells was carried out for 60 min at 37 C The concentration of guanethidine is given as molar concentration Means and S E of 3 expts one is duplicate two in triplicate

the method of Weissbach (1961) as described by Jansson (1970) 5 hydroxytryptamine creatinine sulphate was used as standard

Reagents and drugs
Deionized and twice redistilled water was used throughout All reagents were commercially available analytical grade products n butanol and n heptane were washed according to Weissbach (1961) Drugs Guanethidine sulphate (Ismelin® Ciba AG) 5 hydroxytryptamine creatinine sulphate and 5 hydroxytryptophan (Fluka AG) prenylamine (Segontin® lactate Hoechst AG) reserpine (Sigma Chemical Co) imipramine HCl amitriptyline HCl and cocaine HCl (Medica Ltd) dl amphetamine sulphate (University Pharmacy) FCCP (carbonyl cyanide p-trifluoromethoxy phenylhydrazone) was kindly supplied by M Wikstrom MD Dept Clin Chem Univ Helsinki Guanethidine 5 HT and 5 hydroxytryptophan were dissolved in water reserpine in 0 5 citric acid in 50 % ethanol in water and FCCP in absolute ethanol All the other drugs were dissolved in 50 % ethanol in water Drug concentrations and amounts are given in terms of free bases Mast cells leucocytes and mast cell granules were counted in a Buerker chamber The morphology of the granule sediment was checked electron microscopically (Philips EM 300) using conventional methods for fixation embedding sectioning and post staining

Results

Incorporation of guanethidine into pleural and peritoneal cells

At an exogenous concentration of 5×10^{-5} M 0 596 ± 0 065 nmol guanethidine was incorporated into 10^6 mast cells in 60 min at 37° C (mean and S E of 19 expts in duplicate or triplicate) The corresponding figures for leucocytes were 0 195 ± 0 018 nmol/10^6 leucocytes Taking the volume of 10^6 mast cells to be 1 μl this gives a concentration ratio of 12 between intracellular guanethidine and guanethidine in the incubation medium

Incorporation of guanethidine as a function of temperature

The incorporation of guanethidine into isolated mast cells was highly temperature dependent At an exogenous concentration of 5×10 M the incorporation of

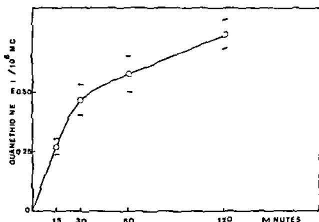


Fig 1 Incorporation of guanethidine into mast cells as a function of time The concentration of guanethidine was 5×10 M incubation temperature 37 C Ordinate uptake of guanethidine expressed as nmol/10⁶ mast cells (MC) Abscissa Incubation time in minutes Means and S E of 2 expts incubated and determined in triplicate

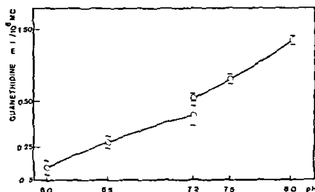


Fig 2 Incorporation of guanethidine into mast cells as a function of pH The concentration of guanethidine was 5×10^{5} M incubation temperature 37 C and incubation time 60 min pH was adjusted by phosphate buffer (130 mM) between pH 6 0 and 7 2 Tris HCl buffer was used between pH 7 2 and 8 0 Ordinate Uptake of guanethidine expressed as nmol/10⁶ mast cells (MC) in logarithmical units Abscissa pH of the medium expressed in pH units Means and S E of 2 expts incubated and determined in triplicate

guanethidine at 4° C and 23° C was 20 and 35 %, respectively of the incorporation at 37° C (Table I)

Incorporation of guanethidine as a function of the exogenous concentration

At an exogenous concentration of 5×10^{6} M barely detectable amounts of guanethidine with the methods used accumulated into the mast cells in 60 min at 37° C With increasing exogenous concentration the amount of guanethidine accumulated increased reaching 1 15 ± 0 111 nmol/10⁶ mast cells in 60 min at $2\,5\times10^{4}$ M (Table II)

Incorporation of guanethidine as a function of time

Guanethidine was rapidly incorporated into isolated mast cells at an exogenous concentration of 5×10 M at 37° C The amount incorporated in 15 min was 47 % and in 30 min 80 % of the amount incorporated in 60 min of incubation (Fig 1) Incubation for 120 min increased the amount to only 28 % above the 60 min

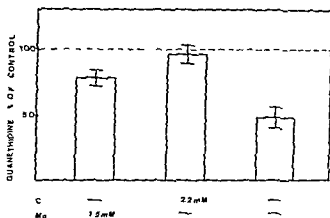


Fig 3 Effect of Calcium and Magnesium chloride on the incorporation of guanethidine into mast cells Incubations were carried out at 37 C for 60 min with guanethidine at 5×10^{-5} M in medium supplemented with both cations (control) with calcium or magnesium alone or with both ions omitted Ordinate Uptake of guanethidine expressed as % of control which was set at 100 % Figures beneath abscissa indicate the ion present in medium and its concentration in mM Means and S E of 2 expts incubated and determined in triplicate

Incorporation of guanethidine as a function of pH

The pH of the medium had a marked effect on the incorporaion of guanethidine A 7 fold increase in the amount incorporated was registered when the pH of the medium was increased from 6 0 to 8 0 At pH 7 2 the incorporation was 25 % higher in a medium buffered with Tris HCl than in a medium buffered with phosphate buffer (Fig 2)

Effect of Ca and Mg ions on the incorporation of guanethidine

The amount of guanethidine incorporated at 5×10^{-5} M at 37° C decreased to 49 % of the control level with the omission of both Ca and Mg ions from the medium

TABLE III Effect of some drugs on the incorporation of guanethidine into isolated mast cells Incorporation with drugs present As of control

Drug added	Concentration	Incorporation of control
Prenylamine	10^{-}	15 3 ± 3 1
Amitriptyline	10^{-4}	17 0 ± 3 3
Imipramine	10^{-}	17 1 ± 3 4
Cocaine	10^{-}	30 3 ± 7 2
Tyramine	10^{-}	68 6 ± 6 6
Amphetamine	10^{-}	98 2 ± 7 8
Imipramine	10^{-}	14 5 ± 2 6
Prenylamine	10^{-}	39 5 ± 2 9
Tyramine	10^{-4}	79 2 ± 3 2
NaCN	10^{-}	89 1 ± 6 1
NaCN	10^{-5}	108 ± 9 9
Ouabaine	10^{-}	103 ± 5 2
FCCP	$3\times10^{-}$	59 6 ± 2 3
FCCP	$3\times10^{-}$	10? ± 9 2

Incubations at 37 C under agitation Drugs were added 15 min before the addition of guanethidine and incubations were then continued for a further 60 min Appropriate amounts to drug vehicle was added to controls Guanethidine concentrat on $5\times10^{-}$ M Means and S E of 1 expts each incubated and determined in triplicate

TABLE IV Effect of 5 HT and 5 HTP on the incorporation of guaneth d ne into isolated mast cel s Incorporation as of control

Drug	Concentration	of control level
5 HT	6 × 10 ⁴ M	20 2 ± 0 8
5 HT	6 × 10⁻⁵ M	44 1 ± 4 1
5 HT	1 2 × 10⁻ M	89 7 ± 2 9
5-HTP	7 × 10	103 ± 6 9

Incubations were carried out at 37 C under agitation 5 HT was added 15 min and 5 HTP 60 min before the addition of guanethid ne whereafter the incubation was continued for a further 60 m n Guanethidine concentration 5 × 10⁻⁶ M Means and S E of 4 expts each incubated and determined in triplicate

TABLE V Effect of reserpine and reserpi ie solvent on the incorporat on of guanethidine into isolated mast cells Incorporation expressed as nmol/10 mast cell

Drug added	Concentration	Guanethid ne nmol/10⁶ mast cells
None	—	0 765 ± 0 073
Reserp ne solvent	5 µl/ml	0 544 ± 0 028
Reserp ne	5 × 10⁻⁶ M	0 55⁹ ± 0 043

Incubations at 37 C fo 60 min under agitation Guanethid ne concentration 5 10⁻ M Reserp ie was d ssolved in 0 5 citr c acid in 50 ethanol in water Both drugs and reserpine sol ent were added simultaneously to the medium Means and S E of 2 expts incubated and determined n tripl cate

TABLE VI Release of guanethidine from preloaded mast cells upon reincubation in drug free medium

	0 min	20 min	40 min	60 min
Total amount recovered (o)	100 ± 7 5	94 4 ± 7 5	94 9 ± 8 2	88 3 ± 3 9
releas d	7 4 ± 3 1	13 6 ± 4 3	27 5 ± 3 6	2 4 ± 4 7

Isolated mast cells were incubated at 37 C for 60 m n w th guaneth dine 5 10 M wa hed 3 times and resuspended n f esh med um Th incub t on v as th n ontinued for another 60 m n at 37 C Samples v ere taken at 0 20 40 and 60 min Guaneth d ne was determined from both pellets and supe n tants Means and S E of 4 expt in tripl cate The total amounts of guanethidin recovered at d fferent times are expressed as of the total amo int at ze o time RELEASED = the amount of guanethid ne reco er d f om the supern tants n pe cent of TOTAL AMOUNT RECOVERED (p ll t + supe natant)

(Fig 3) Omission of only Ca ions caused a drop to 78 % of the control level while omission of only Mg ions did not affect the amount of guanethidine incorporated

Effect of drugs on the incorporation of guanethidine

Prenylamine amitriptyline and imipramine were potent inhibitors of guanethidine incorporation into mast cells (Table III) At 10⁻⁶ M imipramine decreased the amount incorporated to 15 % of the control level being the most potent of

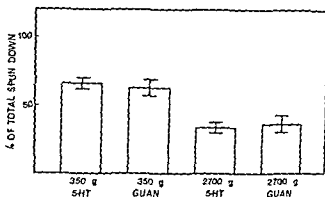


Fig 4 Distribution of guanethidine and 5 HT in subcellular fractions of mast cells previously incubated for 60 min at 37 C with guanethidine at 5×10^{-5} M Fractions collected as described in Materials and Methods Ordinate Amount of 5 HT and guanethidine expressed as % of total spun down represented by the sum of the amount found in the $350 \times g$ and $2700 \times g$ fractions which was set as 100 % Figs beneath abscissa indicate fractions 5 HT and guanethidine respectively Means and S E of 5 expts determined in duplicate

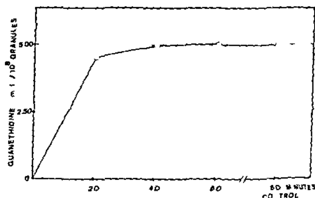


Fig 5 Incorporation of guanethidine into mast cell granules Incubations were carried out at 23 C in 0 32 M sucrose pH 6 9 Ordinate Uptake of guanethidine expressed as nmol/10^9 granules Abscissa Incubation time in minutes Curve represents uptake with 5 HT at 3×10^{-5} added to the medium the control of this experiment (without 5 HT) is indicated by the dotted line Means of 2 expts incubated and determined in triplicate

tested in this respect Cocaine and tyramine were less effective in reducing the incorporation of guanethidine Reserpine amphetamine ouabaine and NaCN had no effect on the incorporation nor did FCCP at 3×10^{-6} M At 3×10^{-5} M FCCP decreased the incorporation to 60 % of the control level

5 HT was a potent inhibitor of the incorporation of guanethidine (Table IV) At equimolar concentrations 5 HT decreased the incorporation of guanethidine to 20 % of the control level and at 6×10^{-6} M the corresponding figure was 44 % 5 hydroxytryptophan at 7×10^{-5} M did not affect the incorporation of guanethidine

Others factors affecting the incorporation of guanethidine

Bovine serum albumine in the medium in concentrations up to 10 mg/ml did not affect the incorporation of guanethidine Ethanol at concentrations higher than 2 % had an inhibitory effect on the incorporation At 0 5 and 1 0 % no effect was seen while ethanol at 0 01 to 0 1 % accelerated the uptake by some 15 % The solvent used for dissolving reserpine (0 5 % citric acid in 50 % ethanol in water) at 5 µl/ml reduced the incorporation of guanethidine to 75 % of the control level (Table V)

TABLE VII Effect of reincubation of isolated mast cell granules preloaded with guanethidine

	0 min	20 min	40 min	60 min
Guanethidine remaining as	100 ±2 9	99 8±4 7	102 ±3 7	104 ±7 7

Isolated mast cell granules were incubated for 60 min at 23 C in 0 32 M sucrose pH 6 9 with guaneth dine 5×10⁻ M After incubation granules were washed twice with fresh sucro e and resuspended in sucrose whereafter incubation was continued for a further 60 min Samples were taken at 0 20 40 and 60 min and after centrifugation granular pellets were assayed for guane th d ne 0 min was set as 100 Means and S E of 2 expts incubated and determined in tripl cate

Subcellar location and binding of guanethidine

The subcellular distribution of guanethidine incorporated into mast cells was in close correlation to the distribution of endogenous 5 HT After freezing and thawing and differential centrifugation of a mast cell population previously incubated with guanethidine 63 5 % of the total amount of the drug spun down was located in the heavy (350×g) fraction and the remaining 36 5 % was located in the granular (2700×g) fraction

The corresponding figures for endogenous 5 HT were 66 0 and 34 0 % respectively (Fig 4) The final supernatant held 10 % of the total amount of guanethidine recovered

Upon reincubation at 37° C of mast cells previously incubated with guanethidine in drug free medium 7 % of the total amount of the drug recovered was found in the incubation supernatant at zero time (Table VI) The corresponding figures at 20 40 and 60 min of reincubation were 14 28 and 25 % respectively The total amount of guanethidine recovered after 60 min of reincubation was 88 3 % of the amount recovered at zero time

Incorporaton of guanethidine into isolated mast cell granules

At an exogenous concentration of 5×10 M guanethidine was rapidly incorporated into isolated membrane free mast cell granules incubated in 0 32 M sucrose pH 6 9 at 23° C A saturation level was reached in less than 40 min (Fig 5) 5 HT at 6×10 did not affect the incorporation of guanethidine to any notable extent The binding of guanethidine to the granules was firm since no release took place upon reincubation of granules in drug free sucrose (Table VII) Surprisingly high amounts of guanethidine accumulated in isolated mast cell granules The amount incorporated increased linearly when plotted arithmetically as the ordinate against the exogenous concentration as logarithmic abscissa in the range 5×10^{-7}–5×10 M Mean and S E of the experiments incubated at 5×10^{-5} M and determined in triplicate was 6 16±0 71 nmol guanethidine/10^9 granules in 30 min at 23° C

Discussion

According to the present results the incorporation of guanethidine into isolated rat peritoneal mast cells *in vitro* was characterized by the following features

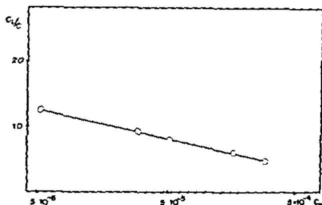


Fig 6 Relationship between concentration of guanethidine in mast cells and final concentration in the medium The final concentration in the medium (C_o) is plotted logarithmically as abscissa against the ratio of concentration of guanethidine in mast cells (C_i)/(C_o) plotted arithmetically as ordinate Means of 2 expts. incubated and determined in triplicate

1 The incorporation was highly temperature dependent A Q_{10} value of 1 91 was calculated from the amounts incorporated at 37° C and 23° C respectively

2 *The incorporation was pH dependent The accelerated incorporation at* alkaline pH probably in part reflects an increase in lipid solubility of the drug due to the increase in the mono-ionized form at pH 8 0 However as 98 % of guanethidine at pH 7 0 is in the di-ionized form (Boullin 1966) it seems unlikely that the decreased incorporation observed at acid pH could be explained solely by a decrease in the lipid solubility of guanethidine

3 The incorporation was dependent on Ca ions It was observed that Mg ions could in part replace Ca ions in the activation of the uptake mechanism. It was interesting to find that these ions which play an important role in neurotransmission processes (see *e g* Douglas 1968) are involved not only in the uptake of 5 HT by mast cells (Jansson 1970) but also in the incorporation of guanethidine

4 The incorporation was fairly rapid An initial rate of uptake of 0 018 nmol/10^6 mast cells/min at 37 C was calculated from the amounts incorporated in 15 min at an exogenous concentration of 5×10 M The uptake did not however increase with time in a linear fashion but a suggested saturation level was reached in 180 min.

5 The incorporation of guanethidine satisfied the criteria of the Michaelis Menten equation when the concentration in the medium (C_o) was plotted against the concentration in the medium divided by the initial rate of uptake a fairly linear relationship was found (Lineweaver and Burk 1934)

6 The incorporation took place against a concentration gradient The intracellular concentration of guanethidine (C_i) already exceeded the extracellular concentration after 3 min at 37° C The relationship between the ratio C_i/C_o and the exogenous concentration (C_o) was straight and the C_i/C_o ratio increased as the C_o values decreased (Fig 6)

7 The incorporation of guanethidine showed saturation characteristics An extrapolation of the C_i/C_o ratio (Fig 6) to zero gives a theoretical intracellular saturation level of 3×10^{-3} M which equals a C_i/C_o ratio of more than 60

These results are compatible either with active transport due to a membrane carrier mechanism or with extensive intracellular binding of guanethidine As will be discussed below guanethidine does become bound intracellularly The incorporation of guanethidine into mast cells showed additional features however which strongly suggest an active transport across the cellular membrane The incorporation was decreased by FCCP suggesting an energy dependent uptake mechanism Further the incorporation was antagonized by the same type of drugs which are known to inhibit the active uptake of amines by adrenergic neurones (see Carlsson and Waldeck 1966 Euler 1969) by mast cell (Day and Stockbridge 1964 Jansson 1970) by platelets (Paasonen 1968) and by central monoaminergic neurons (Carlsson 1970)

It is suggested that drugs like imipramine prenylamine and cocaine all inhibit the "membrane pump for amine uptake The results obtained thus suggest that guanethidine is taken up into mast cells by an active membrane mechanism and that this mechanism may be identical to the 5 HT pump in the membrane of mast cells since 5 HT effectively depressed the uptake of guanethidine

Reserpine did not inhibit the incorporation of guanethidine into mast cells This can be explained by the fact that the incorporation of reserpine into these cells is due to a passive process (Gripenberg Jansson and Partanen 1972) and thus reserpine does not interfere with the active component in the uptake of guanethidine

Knowing that the subcellular location of histamine and 5 HT in mast cells is almost exclusively granular (Lagunoff *et al* 1964 Thon and Uvnäs 1966 Jansson 1970 b) one can take 5 HT to be a granular marker substance The similar distribution of 5 HT and guanethidine in subcellular fractions of mast cells therefore indicates a mainly granular location of guanethidine These results are contrary to those obtained earlier (Boullin and O Brain 1969) indicating that in human platelets guanethidine does not become bound to the amine storing organelles Da Prada and Pletscher (1969 a b) on the other hand reported that in rabbit platelets reserpine is located in the amine storing organelles and recently it was reported that in mast cells reserpine in mainly located in the granules (Gripenberg and Jansson 1971) It therefore seems that in mast cells guanethidine and reserpine become incorporated into the same subcellular organelles

Guanethidine is firmly bound to mast cell since upon reincubation of pre loaded cells 75 % of the total amount recovered was cell bound after 60 min Guanethidine does not seem to be metabolized by mast cells *in vitro* to any great extent judging from the fact that after reincubation in a drug free medium of pre loaded cells 95 % of the theoretical amount was recovered after 40 min of reincubation These results are similar to those obtained by Boullin and O Brien 1969) with human platelets

The incorporation of guanethidine into isolated membrane free mast cell granules seems to be a passive event since 1 the incorporation increased almost linearly with increasing exogenous concentration 2 it was rapid at 23 C reaching saturation level in 40 min and 3 was not affected by high concentrations of 5 HT These re

sults closely resemble those on the incorporation of 5 HT and reserpine into isolated mast cell granules both considered to be passive (Jansson 1971, Gripenberg Jansson and Partanen 1972)

Mast cells have been extensively and successfully used as a model for the mono aminergic nerve terminal in amine uptake and release studies (Green 1966 Jansson 1970) The present results therefore confirm previous observations that guanethidine is taken up into the sympathetic neurone by an active membrane transport mechanism Intracellularly guanethidine becomes incorporated into the amine storing organelles Guanethidine thus interferes with neuronal amine dynamics by at least two distinct mechanisms firstly by decreasing the uptake of amines, probably by a competitive mechanism and secondly by interfering with the granular amine storing mechanism

It has been shown (Jensen Holm and Juul 1970, Eranko and Eranko 1971) that guanethidine induces distinct morphological changes in the sympathetic ganglia of the rat It has also been shown that guanethidine interferes with the oxidative phos phorylation *in vitro* (Malmquist and Oates 1968) and that the drug becomes con centrated in the sympathetic ganglia (Juul and Sand 1971) and induces pronounced swelling of the mitochondria in the nerve cells of rat superior cervical ganglia (Jen sen Holm and Juul 1971) It is not known whether the morphological effects are due to extensive incorporation or whether the changes are due to an altered metabolism induced by guanethidine A closer study of the effect of guanethidine on both cellular metabolism and amine dynamics is in progress in our laboratory

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TABLE II The excretion of noradrenaline in supine position and during tilting +70

Case no	Noradrenaline ng/min		Diuresis after 1 h ml	
	supine	tilted 70	supine	tilted 70
1	40 1	45 9	270	200
2	35 2	29 4	280	130
3	19 3	18 3	210	180
4	26 3	33 4	220	190
5	45 0	51 5	380	460
6	35 0	35 8	280	450
7	26 6	28 3	200	150
8	19 8	22 5	320	200

min The maximal work performed varied from 400 to 900 kpm/min The working capacity was within the normal range for the age (Strandell 1964)

The mean heart rate increase in response to tilting head up 70 for 60 min was 9 beats/min and did not differ from the mean increase during 8 min standing (8 beats/min) The orthostatic pulse response did not differ from that of young subjects (Strandell 1964)

The excretion of noradrenaline during the supine and tilting periods are shown in Table II The mean excretion of noradrenaline in supine was 30 9 ng/min (S D ± 9 4) and during standing 33 1 ng/min (S D ± 11 2)

The diuresis values given in table II did not change significantly from supine to standing position

Discussion

The circulatory findings in the present material are all in agreement with those found in healthy old man (Strandell 1964) The subjects studied could thus be considered circulatory normal

With advancing age progressive structural changes occur in the cardiovascular system with increase in thickness and rigidity of the walls of arteries and veins (Bourne 1961) A higher peripheral resistance and a higher mean arterial pressure follows The maximal heart rate decreases and the heart volume increases even in relation to total hemoglobin (Sjostrand 1949) Drop in stroke volume in response to changes in body position from supine to erect is less marked in elderly than in young subjects and the decrease in cardiac output is less pronounced (Strandell 1964)

Reduced reflex circulatory compensation to postural changes has been reported in aged (Norris Shock and Yiengot 1953 Gross 1969) Total lack of baroreceptor response without symptoms is not an uncommon finding in healthy old people (Appenzeller and Descarries 1964)

Under basal conditions the excretion of noradrenaline is the same in young and old subjects (Kärki 1956) The present investigation was performed in the morning

and the excretion values are comparable to those found by Karki during the first day time eight period (Karki 1956) The response to tilting does not seem to have been studied earlier in aged people

In the present study no orthostatic fall in systolic blood pressure was seen immediately after assumption of standing position This is in contrast to the finding in young subjects and might be due to the less variability in venous return which secondarily is probably due to the more rigid capacitance vessels in the aged (Bourne 1961) The lack of orthostatic pressure drop could well explain why no increased vasomotor nerve activity occurs in response to changes of body position and why urinary excretion of noradrenaline remains unchanged

In younger individuals the orthostatic pressure fall is compensated by a marked increase of the peripheral resistance which requires an increased activation of the noradrenergic vasomotor nerves This is mirrored by a fourfold increase of the urinary excretion of noradrenaline (Sundin 1956)

In young subjects tilting is often followed by vasovagal syncope shortly after the change to upright position In these cases extremely high values of noradrenaline has been found in plasma with a peak response just prior to the syncope (Vendsalu 1960) In the present investigation (here were no orthostatic complaints) syncope occurred in one of the subjects after 55 min standing However the excretion of noradrenaline showed no increase in this case

In patients with postural hypotension (where even the resting urinary catechol amine output is low) the noradrenaline excretion does not increase in response to tilting (Luft and Euler 1953) It seems obvious that in elderly subjects a similar lack of noradrenaline increase at tilting may be considered a normal consequence of an altered reflex circulatory adaptation Further information is required in order to find out at which age this change occurs

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Drug Effects on Pre- and Post-branchial Blood Pressure and Heart Rate in a Free-swimming Marine Teleost, Gadus morhua[1]

By

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Abstract

HELGASON S S and S NILSSON *Drug effects on pre and postbranchial blood pressure and heart rate in a free swimming marine teleost Gadus morhua* Acta physiol scand 1973 *88* 533—540

Drug effects on pre and post branchial blood pressure and heart rate have been simultaneously and continuously registrated in free swimming cod (*Gadus morhua*) Adrenaline noradrenaline and phenylephrine increased blood pressure presumably by increasing systemic vascular resistance by alpha adrenergic stimulation The heart rate was decreased by the same drugs obviously by a baro-receptor reflex with the efferent inhibitory cholinergic fibres running in the vagus nerve Yohimbine decreased the blood pressure and blocked the effect of adrenaline The drop in blood pressure may be due to blockade of a normally acting vaso-constrictor effect on the systemic vessels Pre and post branchial blood pressure was reduced by isoprenaline and this effect was reversed by propranolol suggesting presence of adrenergic beta receptors in the systemic vascular bed Propranolol also caused a drop in heart rate and post branchial blood pressure while the pre branchial blood pressure was increased This again could be due to abolishment of a normally acting adrenergic influence on the heart and branchial vessels

In teleost fish, the blood leaving the heart by the ventral aorta passes the respiratory capillaries of the gills and is collected into the dorsal aorta which drains the blood from the gills Branching arteries from the dorsal aorta distribute the blood to the capillaries of the various tissues The blood is then recollected into the veins and returned to the sinus venosus of the heart Thus the respiratory and systemic capillaries are coupled in series with the heart forming one single circuit (Fig 1)

The vascular resistance of the gills brings about a large decrease in pressure from ventral to dorsal aorta The next abrupt drop in pressure occurs in the systemic capillaries and the pressure of the blood returning to the heart is close to zero An integrated regulation of the diameter of the branchial and systemic vessels is an

KEY WORDS *Gadus morhua* teleost fish circulation baroreceptors adrenergic drugs autonomic fibres

[1] Part of this work was presented at the meeting of the Scandinavian Physiological Society in Uppsala Acta Phys ol Scand 1973 *87* 8 A

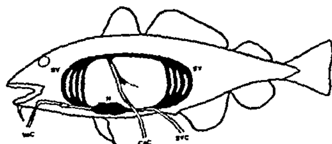


Fig 1 *Gadus morhua* Simplified diagram of circulation Legend BV branchial vessels CAC celiac artery catheter H heart SV, systemic vessels SVC spermatic vein catheter VAC ventral aorta tipped catheter

essential factor in maintaining an adequate blood flow the other main factor being the regulation of cardiac output

Practically all work on branchial resistance changes in teleosts has been carried out on isolated gill preparations (Keys and Bateman 1932 Östlund and Fange 1962 Steen and Kruysse 1964 Reite 1969 Richards and Fromm 1969) In this kind of preparations it is found that the branchial vessels are dilated by catechol amines and constricted by acetylcholine

Information on systemic vascular resistance is also mainly obtained from isolated preparations either on perfused vascular beds (Reite 1969 Stray Pedersen 1970 Nilsson 1972 a) or on isolated vessels (Kirby and Burnstock 1969) It is suggested that there is a cholinergic and an adrenergic vasoconstrictor innervation of the large arteries in teleosts while the vascular bed is supposed to be innervated by cholinergic vasoconstrictor nerves only (Burnstock 1969) No inhibitory effects of catechol amines have been detected in large arteries (Kirby and Burnstock 1969)

Cardiac function has since long been extensively studied *in vitro* and Gannon and Burnstock (1969) demonstrated in the trout adrenergic vagal excitatory fibres to the heart in addition to the cholinergic vagal inhibitory fibres A vagal cholinergic inhibitory tonus on the teleost heart may exist (Randall and Stevens 1967)

In recent years information has been obtained from *in vivo* experiments involving measurements of blood flow blood pressure and heart rate in intact fish There is no evidence for an adrenergic vasoconstrictor tonus in teleosts (Randell and Stevens 1967) For reviews of literature see Johansen (1971) Satchell (1971) and Randall (1970)

Of the relatively few studies involving measurements of pre and postbranchial blood pressure in free swimming teleosts most have been done on fresh water teleosts (Stevens and Randall 1967 Holeton and Randall 1967) with few exceptions (Saunders and Sutterlin 1971) In spite of earlier information it is still not clear to what extent the regulation of branchial and systemic vascular resistance respectively prevails in setting blood pressure

This study was undertaken on cod *Gadus morhua* a common marine teleost which has been object to cardiovascular and other studies in this laboratory The purpose of this study was to investigate the effects of various drugs interfering with the autonomic nervous regulation of the circulatory system and to evaluate the

respective roles of the branchial and systemic resistances in setting the blood pressure Further to evaluate if any nervous or hormonal influence exists in the systemic and branchial vascular beds and finally to produce further basic information about vasomotor reflexes in teleosts

Materials and methods

Simultaneous and continuous recordings of pre and post branchial blood pressure and heart rate were made from free-swimming un anesthetized cod *Gadus morhua* with a bod weight of 350—2000 g The fish were kept in well aerated streaming sea water at 12 —14 C during the experiments During the implantation of catheters the animals were anesthetized with MS 222 (Sandoz) 50 mg pro liter sea water

Pre-branchial blood pressure was recorded via a tipped heparinized catheter (PE 50) inserted into the ventral aorta through the tongue (Garey 1969)

Post branchial blood pressure was measured via a catheter (PE 50) inserted into the celiac artery An incision was made just behind the right pectoral fin and the celiac artery was freed of surrounding tissues A few cm behind the incision the body wall was punctured by a large hypodermic needle (G 15) which served to introduce the catheter into the body cavity after which the hypodermic needle was withdrawn The catheter was finally secured to the body wall by a piece of rubber on each side and inserted into the celiac artery Catheterization of the permatic (ovaric) vein for injection of drugs was performed in a similar manner The incision was then sutured and the animals allowed to recover from the anesthesia in the experimental aquaria for at least two hours prior to any injection of drugs

The arterial catheters were attached to Statham P 23 pressure transducers and recordings were made on a GRASS Polygraph mod 7 Continuous registration of heart rate was obtained from the blood pressure signal triggering a tachograph preamplifier (7 P 4)

Bilateral vagotomy was carried out in a few animals Incisions about 1 cm long were made behind the opercula and the intestinal and cardiac vagus trunks on both sides were cut

The following drugs were used in the experiments
Acetylcholine chloride L-adrenaline bitartrate atropine sulphate hyoscine hydrobromide DL-isoprenaline hydrochloride L-noradrenaline bitartrate L-phenylephrine hydrochloride propranolol hydrochloride yohimbine hydrochloride The drugs are referred to as μg or mg/kg b wt of these salts

Results

Simultaneous recording of pre and post branchial blood pressure and heart rate was started immediately after the operation and continuously followed throughout the experiment Blood pressure and heart rate being low at first steadily increased during the first half hour as the anestetic effect was wearing off (Houston *et al* 1971) reaching steady state values These values were maintained until the start of drug injections and will be referred to as normal values The breathing movements being irregular at first also stabilized during the first half hour

Mean normal values ± S E for the 22 animals used in the experiments were as follows pre branchial blood pressure (ventral aorta) 58±2/49±2 cm H_2O (44±2/37±2 mm Hg) post branchial blood pressure (celiac artery) 39±3/33±3 cm H_2O (30±2/25±2 mm Hg) and heart rate 53+1 beats per minute This means a pressure drop over the gills of about 30—35 per cent

DRUG EFFECTS

Adrenaline (0 05—20 μg/kg) caused marked increase in both pre and postbranchial blood pressure This increase was usually followed by a distinct bradycardia (Fig

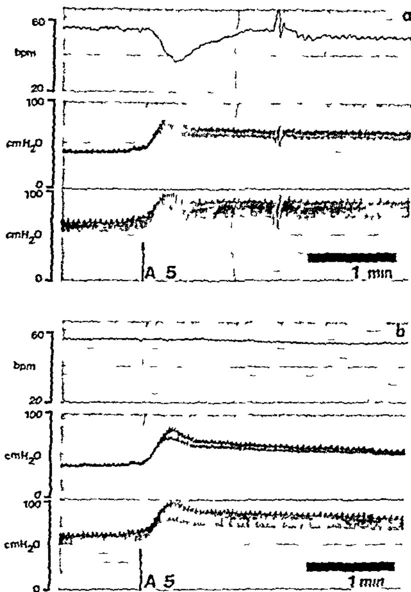


Fig 2 Gadus morhua Upper channel heart rate middle channel postbranchial blood pressure lower channel pre branchial blood pressure At arrow injection of adrenaline 5 µg/kg Note the marked bradycardia in *a* Between *a* and *b* is 30 min and hyoscine 4 mg/kg) has been injected

2 a) Noradrenaline (0 5—50 µg/kg) and phenylephrine (10—100 µg/kg) caused similar effects Isoprenaline (0 5—20 µg/kg) on the other hand produced a decrease in pre and post branchial blood pressure while the heart rate remained unaffected or increased slightly (Fig 4) Pulse pressure appeared to be increased by isoprenaline adrenaline and noradrenaline (Figs 2 and 4)

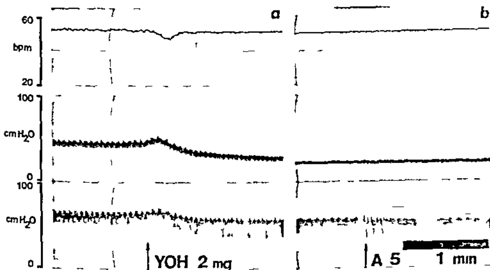


Fig 3 *Gadus morhua* Upper channel heart rate middle channel postbranchial blood pressure lower channel pre branchial blood pressure In *a* injection of yohimbine (2 mg/kg) in *b* injection of adrenaline (5 μg/kg) Between *a* and *b* is 40 min

Yohimbine produced a sustained decrease in pre and post branchial blood pressure the effect lasting for several hours (Fig 3 a) The heart rate decreased for the first minute after the yohimbine injection but was soon restored (Fig 3) Adrenaline (5—10 μg/kg) injected after yohimbine (2 mg/kg) never increased blood pressure but sometimes caused a hardly visible decrease the heart rate unaffected (Fig 3 b) Partial blockade of the adrenaline effect could be seen after 1 mg/kg of yohimbine

Propranolol (2 mg/kg) produced an increased pre branchial blood pressure and decreased post branchial blood pressure and heart rate (Fig 4 b and c) The effect of isoprenaline was reversed and a slight increase in blood pressure could be seen with isoprenaline after propranolol (Fig 4 c) The effect of propranolol lasted for many hours being more or less irreversible The blood pressure increasing effect of adrenaline was unaffected by propranolol

High doses of acetylcholine (20—50 μg/kg) caused arrest of the heart thus lowering blood pressure Lower doses of acetylcholine gave very irregular results but often caused an initial decrease in pre and postbranchial blood pressure followed by an increase above the resting level The effects on heart rate were slight with low doses of acetylcholine

Atropine or hyoscine (4 mg/kg) caused transient increase in arterial blood pressure together with a likewise transient decrease in heart rate Lower doses of these drugs (2 mg/kg) produced smaller effects on blood pressure and a slightly increased heart rate

Adrenaline (5 μg/kg) after atropine or hyoscine (4 mg/kg) still produced an increase in blood pressure while the decrease in heart rate was strongly reduced or

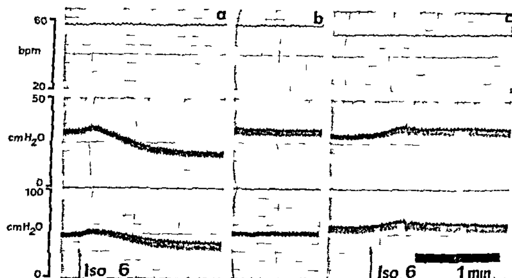


Fig 4 *Gadus morhua* Upper channel heart rate middle channel postbranchial blood pressure lower channel pre branchial blood pressure In *a* and *c* injections of isoprenaline (6 μg/kg) Between *a* and *b* is 10 min Between *b* and *c* is 35 min and propranolol (2 mg/kg) has been injected Note increase in pre branchial blood pressure and decrease in postbranchial blood pressure and heart rate after propranolol

abolished (Fig 2 a and b) Bilateral vagotomy also abolished the bradycardia obtained after adrenaline injection although the effect of adrenaline on blood pressure subsisted

Discussion

Adrenaline and noradrenaline have been shown by several authors to decrease vascular resistance of the gills in teleost fish (Keys and Bateman 1932 Östlund and Fange 1962 Steen and Kruysse 1964 Reite 1969 Richards and Fromm 1969) If only changes in branchial resistance were taking place after these drugs in *intact* fish one would expect an increased post branchial and a decreased pre branchial pressure to occur In the present experiments however an increase in arterial pressure both pre and post branchially was recorded This could depend on an increased cardiac output and/or an increased systemic vascular resistance In the isolated plaice (*Pleuronectes platessa*) heart adrenaline or noradrenaline produced positive inotropic and chronotropic effects (Falck *et al* 1966) No increase in heart rate could account for the increase in blood pressure since marked bradycardia occurs after adrenaline or noradrenaline Propranolol which could be expected to block the effects of catecholamines on the heart (Falck *et al* 1966) does not inhibit the pressure rise seen after adrenaline or noradrenaline On the other hand the alpha adrenergic blocking agent yohimbine produces such an inhibition It is therefore concluded that the pressure increasing effect of adrenaline or noradrenaline as well as phenylephrine is mainly due to increased systemic vascular resistance caused by stimulation of adrenergic alpha receptors In salmonid (*Onchorynchus kisutch* and *O nerka*) another alpha receptor blocking agent

phenoxybenzamine proved to effectively block the pressure increasing effects of adrenaline However phenoxybenzamine *per se* did not affect the blood pressure in these species (Randall and Stevens 1967)

The decrease in pre and post branchial blood pressure seen after yohimbine in the present experiments could be explained by assuming that alpha receptor blockade causes a decrease of a noradrenergic nervous tonus or abolishe the effects of circulating catecholamines on the systemic vessels Noradrenergic innervation of blood vessels has been demonstrated in the trout and eel (Kirby and Burnstock 1969) and the cod (Nilsson 1972 b) It may also be concluded that in response to catecholamines the branchial vessels play little role in controlling blood pressure compared to the systemic vessels

The decrease in pre and post branchial pressure following isoprenaline indicates presence of adrenergic inhibitory receptors in the systemic vessels These receptors may be of the beta type since isoprenaline is the only catecholamine able to produce this effect and since propranolol reversed the response In large arteries from eel and trout however no inhibitory effects of isoprenaline were detected (Kirby and Burnstock 1969)

Propranolol increases the pre branchial and decreases the post branchial blood pressure and heart rate Again this suggests that the branchial vessels are innervated by normally tonically active adrenergic fibres the effect of which is abolished by propranolol The level of circulating catecholamines may also normally affect the degree of branchial vascular tonus The same may be true for the heart where tonically active noradrenergic fibres or circulating catecholamines could produce an elevated heart rate Noradrenergic excitatory fibres to the trout heart have been demonstrated (Yamauchi and Burnstock 1968 Gannon and Burnstock 1969)

The failure of adrenaline noradrenaline and isoprenaline to produce but a weak increase in heart is striking The effect on stroke volume may also be small since no visible increase in pulse pressure occurs with adrenaline after yohimbine In the isolated plaice (*Pleuronectes platessa*) heart these drugs have been shown to cause both positive inotropic and chronotropic effects (Falck *et al* 1966) and therefore in the cod cardiac output may be increased to some degree after the catecholamines although this is not shown in the present investigation It must be pointed out that great species differences between teleosts may exist in this and several other respects

The strong transient decrease in heart rat occuring during the pressor response induced by adrenaline and noradrenaline is obviously a reflex as pointed out earlier (Randall and Stevens 1967) The heart rate is decreased presumably by an increased vagal cholinergic tonus on the heart since the effect is abolished by atropine and hyoscine (see also Randall and Stevens 1967) and by vagotomy The afferent pathway of the reflex arch may originate from baroreceptors in the pseudo branchs (Laurent 1967) or elsewhere (Mott 1951) Experiments are in progress in this laboratory to further elucidate the role of baroreceptors in regulating the blood pressure and heart rate in teleosts

The small increase in heart rate seen after atropine or hyoscine indicates tl

vagal tonus in the free swimming cod is small The initial decrease of heart rate after larger doses of these drugs may be due to acetylcholine like effects but further work is necessary to clarify the inconsistent effects of acetylcholine and the cholinergic part of circulatory regulation in the cod

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Acta physiol scand 1973 88 541—550

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Mucosal Lesions in the Small Intestine of the Cat during Low Flow

By

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Abstract

Ahren C and U Haglund *Mucosal lesions in the small intestine of the cat during low flow* Acta physiol scand 1973 *88* 541—550

The mucosal changes produced at the villous tips of the cat small intestine during a mimicked shock situation (regional hypotension at 30 mm Hg during nervous vasoconstrictor fibre activation) were examined macro- and microscopically Macroscopically petechial bleedings and ulcerations were regularly seen in the mucosa after a 2 h hypotensive period The microscopic findings were characterized by epithelial lifting of various degrees at the tips of the villi and in some cases by a complete denudation of the villous tips Intraluminal perfusion of a segment with oxygenated saline during the hypotension prevented or greatly reduced the mucosal lesions compared to control segments On the other hand intraluminal perfusion with nitrogenated saline did not change the mucosal lesions as compared with control This strongly indicates that hypoxia *per se* plays an important role in the pathogenesis of the mucosal lesions while the intestinal content (*e g* energy substrates or enzymes) does not appear to be of any great importance It is suggested that the hypoxia is caused by an extravascular shunting of oxygen in the intestinal countercurrent exchanger

Hemorrhagic lesions in the small intestinal mucosa during hemorrhagic shock and experimental intestinal ischemia have been reported in man (*e g* Penner and Bernheim 1939 Klemperer *et al* 1940 Ming 1965 Sorensen and Vetner 1969) in dogs (*e g* Wigger 1950 Lillehei 1957 Chiu *et al* 1970 a) in normal and in germ free rats (Zweifach *et al* 1958 Bacalzo *et al* 1971) and in cats (Haglund and Lundgren 1972 b 1973) However the pathogenesis of the mucosal lesions is still debated They have been ascribed to spasm or intense constriction of the intestinal resistance vessels leading to enough of hypoxia as to damage the tissues (Klemper *et al* 1940 Lillehei *et al* 1967) On the other hand Bounous and coworkers (Bounous *et al* 1964 1967 Bounous 1967) believe that the intestinal content especially the presence of trypsin is of prime importance According to them hypoxia makes the epithelium vulnerable to tryptic digestion but does not *per se* cause the ulcerations

It is however evident from recent studies (Lundgren and Svanvik 1973) that the villous mucosal blood flow is surprisingly little reduced during intestinal hypotension at 40 mm Hg or during intense vasoconstrictor fibre activity

publication (Haglund and Lundgren 1973) an alternative hypothesis regarding the development of the mucosal lesions was therefore presented based on the counter current exchange mechanism in the hairpin vascular loops of the villi (Lundgren 1967) According to this hypothesis the extravascular shunting of oxygen normally occurring at the villous base (Kampp *et al* 1967) becomes far more efficient during hypotension, due to a markedly reduced *linear* rate of blood flow in the villi Thus *in spite of an almost unchanged mucosal blood flow* a severe lethal hypoxia could then develop at the tips of the villi

The aim of the present study was to examine the microscopic appearance of the mucosal lesions in the cat Further the effects of an intraluminal perfusion with oxygenated or nitrogenated saline on the mucosal lesions were studied to find out *which of the two abovementioned factors tissue hypoxia or the intestinal contents* were primarily responsible for the mucosal lesions

Methods

A Operative technique and determination of blood flow Experiments were performed on 9 cats weighing 2 5—5 9 kg anesthetized i v with chloralose (50 mg/kg b w) after induction with ether The cats had been deprived of food for 12 h and had no obvious signs of intestinal infection The details of the operative technique is described elsewhere (Haglund and Lundgren 1972 a) Briefly the abdomen was opened in the midline and 3—9 segments of the small intestine each weighing 7—10 g were isolated from each other beginning from the ligament of Trietz The remainder of the intestinal tract was extirpated together with the spleen and the greater omentum The splanchnic nerves containing the sympathetic nerve fibres to the intestinal segments and to the adrenals were cut and their distal ends were placed on ring electrodes for stimulation (6 Hz 6 ms and 10 mA) An adjustable clamp was placed around the superior mesenteric artery close to the aorta where the artery is not closely emeshed by nerves making it possible to lower the intestinal arterial inflow pressure to any desired level without damaging the nerves (Haglund and Lundgren 1972 a) The cats were heparinized and blood pressure was recorded from the left femoral artery and also from a major branch of the superior mesenteric artery by means of two pressure transducers (Statham P 23) The right femoral artery were cannulated for taking arterial blood samples Venous outflow from the intestinal segments and their lymph nodes was measured by an optical drop recorder unit operating an ordinate writer venous outflow pressure was set to 10 mm Hg The blood was returned to the animal *via* a cannula in the right external jugular vein

In order to maintain a fairly constant arterial pH a bicarbonate solution (10 mekv $NaHCO_3$ per 10 % glucose solution) was infused i v from the start of the anesthesia and throughout the experiment This procedure was checked by repeated determinations of pH in arterial and intestinal venous blood during the experiments (Astrup pH meter 27 with a microelectrode unit) Further 2 ml of a dextran solution (1/3 Rheomacrodex® and 2/3 Macrodex® Pharmacia, Sweden) was given every 30 min to diminish rheological disturbances

The lactic acid concentrations in arterial and intestinal venous blood were determined repeatedly in 3 expts by means of a Boehringer set (Boehringer lactate UV test Boehringer Mannheim) Knowing the lactate concentration difference between intestinal venous and arterial blood and the intestinal blood flow at the time of sampling *the intestinal release of* lactic acid could be calculated in μmol/min × 100 g tissue

B Experimental procedures Experiments were performed on intestinal segments treated in the following way 1 control segments (all animals) 2 segments with luminal perfusion using oxygenated saline throughout the hypotensive period (8 animals 3 segments with luminal perfusion using nitrogenated saline throughout the hypotensive period (8 animals) 4 segments filled with stagnant saline throughout the experiment (6 animals) 5 segments filled with stagnant 5 5 % glucose throughout the experiment (6 animals 6 segments flushed intraluminally with 200—300 ml saline prior to the hypotension period (3 animals) The luminate perfusate was kept at 37 C and the perfusion rate was 20—30 ml/min The segments filled with saline or glucose were tied in both ends The relative positions of the segments along the intestinal tract were randomized in the different experiments



Fig 1 Representative microscopic pictures of the intestinal mucosa illustrating the grading of the mucosal lesions For details see text

The animals were allo ed to re t after finishing the preparation to obtain steady state levels for arterial blood pressure and intestinal blood flow Inte tinal arter al inflow p essu e was then reduced to about 30 mm Hg by means of the adjustable clamp and the st mulation of the splanchnic nerves was started After ɔ—3 h the timulation was stopped and the partial arterial occlusion released The cats were then follo ed during an addit onal 1 h period and the intestinal segments ere emo ed immediately after death and tissue sections fo histolog cal examination were tak n

C Histolog cal te h q e At the end of the exp riments rectangular segments of the n test nal wall about 2 × 4 cm in ize ere rap dly cut out mount d on cork and fix d in 10 % neutral formalin The blocks were further prepared for routine paraffine embedment cut at about 4 μ mounted and ta ned accord g to v Gieson in hematoxyline eosine and with Mc Manus PAS stain The glasses ere coded for histolog c examination

The morpholog al ch nges in the muco a were graded as descr bed b Chiu *t al* (1970 a) into 6 grades wh r g ade 0 means normal mucosa and grade I to V mean in reasing damage of the surface pithelium The pathogno nonic le on for grade I is the development of a subep thel al space at the tips of the ill Th s pace is more ext nded in grade II where also epithelial l fting een In grade III there a ma e pithel al l fting down the s des of the villi and in grade IV the lli are denuded Grade V is characterized by dis ntegration of the lamina propria hemorrl age and ulcerat on

Th diffe ent grades are illustrated in Fig 1 Fo further details see Chiu *et al* (1970 a

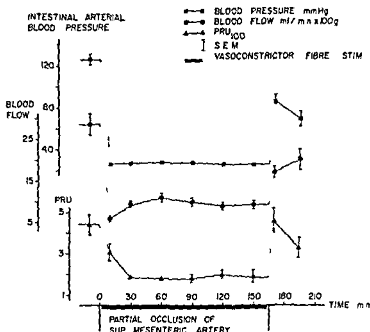


Fig. 2 The effects of a prolonged partial occlusion of the superior mesenteric artery together with a continuous stimulation of the intestinal vasoconstrictor nerve fibres on intestinal blood flow and flow resistance Mean value ± S E (n = 9)

Results

A Hemodynamic observations Fig 2 illustrates intestinal arterial inflow pressure intestinal blood flow and blood flow resistance before during and after a 2 5—3 h regional hypotension with the intestinal arterial inflow pressure kept at 25—30 mm Hg In the prehypotensive control period intestinal blood flow amounted to 28 6 ± 2 7 ml/min × 100 g resistance being 4 4 ± 0 5 PRU_{100} units (mean ± S E n = 9) As shown in Fig 2 intestinal blood flow and more slowly so also the resistance to flow decreased when intestinal perfusion pressure was reduced simultaneously with an activation of the intestinal vasoconstrictor nerve fibres Within 30 min however blood flow resistance came to a fairly stable level blood flow being now 10 ml/min × 100 g implying a fall in resistance to about 40 % of control Upon releasing the partial arterial occlusion a short lasting reactive hyperemia occurred but 5 min later blood flow was returned to levels below the control one (17 1 ± 1 3 ml/min × 100 g) However, arterial blood pressure was now reduced to 86 ± 7 mm Hg and intestinal flow resistance equalled that during control

During the subsequent 20—25 min intestinal blood flow increased in spite of a further reduction in blood pressure, indicating an accentuated reduction in blood flow resistance 30—35 min after releasing the partial occlusion 5 of the 9 animals died The remaining 4 animals were followed during 60 min and during the last

TABLE I Arterial and intestinal veno is blood pH bef re during and afte regional arterial hypotension at approximately 30 mm Hg Mean values ± S E n = 9

	Prehypotensive control period	Intestinal hypotension		Posthypo ten ive control period
		60 min	120 min	
Arterial blood pH	7 34 ± 0 01	7 33 ± 0 03	7 38 ± 0 02	39 _ 0 04
Intestinal venous pH	7.28 ± 0 01	ı 17 ± 0 03	7 14 _ 0 04	?ı 0 04

TABLE II The amount of lactic acid released from th intestine befor during and aft r r ο or al arterial hypotension at approximately 30 mm Hg Mean value ± S E n = 3

	Prehypotensive control period	Intestinal hypotension		Posthypo te isı e control period
		60 min	120 min	?0 min
Release of lactic acid from the intestine µmol/min × 100 g	13 7 ± 4 3	26 7 ± 10 7	26 3 ± 3 3	?4 7 ± 1 8

30 min a progressive fall in blood pressure and in blood flow resistance was seen while blood flow was unchanged

Arterial blood pH was kept fairly constant throughout the experiments as shown in Table I while the intestinal venous pH was reduced during the hypotensive period 20—30 min after releasing the partial occlusion intestinal venous blood pH returned to the prehypotensive control value

Table II presents the results on lactic acid release from the intestine before during and after the regional hypoten.ıon and shows that lactic acid output from the intestine was increased during and after hypotension

B Morphological observations An immediate macroscopic postmortem examination of the control intestinal egments revealed hemorrhagic lesions in 8 of 9 cats In one animal the mucosa was macroscopically as well as microscopically normal both in the control and in the perfused segments and this animal is not included in the results reported below The *macroscopic lesions* varied from petechial bleedings in some animals to mucosal destruction in the entire egment in others No clear-cut macroscopic difference was found between the control segments and the ones perfused with nitrogenated saline In contrast 6 of 7 segments perfused with oxygenated saline appeared macroscopically normal with petechial bleedings in the remaining animal Macroscopic hemorrhagic lesions were also found in the muco.a of all the intestinal segments instilled with stagnant glucose or saline or initially flushed with saline Thus none of these latter procedures eemed to reduce the macroscopic lesions as compared to control

TABLE III Microscopic grading of intestinal control segments and of segments perfused with oxygenated or nitrogenated saline during hypotension Figures denote number of segments Observations were made in 7 expts

	Number of intestinal segments showing mucosal lesions graded as grade				
	0	I	II	III	IV
Control segments			1	4	2
Segments perfused with oxygenated saline	3	2	2		
Segments perfused with nitrogenated saline	2	1		1	3

TABLE IV Microscopic grading of intestinal control segments and of segments filled with glucose saline or washed with saline Figures indicate number of segments Observations were made in 3–7 expts

	Number of intestinal segments showing mucosal lesions graded as grade				
	0	I	II	III	IV
Control segments			1	4	2
Segments filled with glucose			1	2	3
Segments filled with saline		1		3	2
Segments washed with saline before hypotension				1	2

Microscopic grading of the different segments (Methods section C Fig 1) produced the results given in Table III and IV It is evident from Table III that in 6 of 7 cats profound mucosal lesions (Grade III—IV) were found in the control segments On the other hand compared to control perfusion with oxygenated saline improved the mucosal histology in all cats with no or only slight lesions (Grade 0—II) Intraluminal perfusion with nitrogenated saline improved the mucosa compared to control in only 3 of the 7 cats, no difference being seen in 2 while 2 segments showed acceentuated lesions compared with the control segments Table IV presents the results from the microscopic examination of the segments filled with 5 5 % glucose or 0 9 % saline or initially flushed with saline none of these procedures appeared to affect the development of the mucosal lesions

Discussion

In earlier hemodynamic studies of the cat s small intestine hemorrhagic mucosal lesions were regularly found when the regional vasoconstrictor fibres were intermittently stimulated during regional intestinal or hemorrhagic hypotension (Haglund and Lundgren 1972 b 1973) No qualitative difference was observed between these two types of *intestinal hypotension* with respect to intestinal haemodynamics or the

appearance of hemorrhagic mucosal lesions However obvious lesions failed to appear even after prolonged regional hypotension when the intestinal vessels had been deprived of the influence of the vasoconstrictor fibres (Haglund and Lundgren 1972 a) Therefore an experimental model allowing local intestinal hypotension as combined with a continuous regional vasoconstrictor fibre activity was considered suitable for the present series of experiments Furthermore such an experimental model in all probability *mimicks the true situation for the intestine during hemorrhagic* shock

The hemodynamic events recorded in the present study are shown in Fig 2 In spite of the continuous vasoconstrictor fibre stimulation regional hypotension resulted in a pronounced fall in intestinal flow resistance as compared to control After releasing the partial vascular occlusion a general cardiovascular derangement of the experimental animals was seen and more than half of the animals died within half an hour Qualitatively similar findings were reported earlier for regional intestinal and systemic hemorrhagic hypotension (Haglund to be published Haglund and Lundgren 1972 b 1973) For a detailed discussion of the hemodynamic findings see these papers

The macroscopical changes in the intestinal mucosa of the control segments observed in the present experimental model were similar to those reported in the earlier cat studies (Haglund and Lundgren 1972 b 1973) and to tho e on dogs (*e g* *Lillehei 1957*) rats (*e g* Bacalzo 1971) and man (*e g* Ming 1965) The histological appearance of the lesions were identical to tho e in dogs (Chiu *et al* 1970 a) and similar to the ones reported from studies on man (Ming 1965 Sorensen and Vetner 1969) although the latter studies were performed on autopsy material where post mortem autolysis cannot be excluded

Chiu and coworkers (1970 a) proposed a system for histological grading of the mucosal lesions observed in the dog intestine during low blood flow and showed furthermore that the grading was related to the reduction of total intestinal blood flow Using this system it was demonstrated in the present study that luminal perfusion (20—30 ml/min) of intestinal segments (weight 10 g) with oxygenated saline during the hypotension markedly improved the macroscopic as well as the microscopic appearance of the segments as compared to controls (Table III) On the other hand similar perfusions of adjacent segments but using saline equilibrated with nitrogen instead during the hypotension did not significantly reduce the incidence or severity of the mucosal lesions as compared with control segments Neither did luminal flushings with about 300 ml saline performed prior to the hypotension improve the macroscopic or microscopic picture of the mucosa (Table III and IV) It can therefore be concluded that hypoxia in the villi constitutes the key factor in the pathogenesis of the mucosal lesions seen in the cat s intestine after hypotension

There are several other observations in the literature that support the notion that hypoxia can develop rapidly in the villi during low blood flow states Thus the mitochondria are the first of the organelles to show damage in states of low blood

flow or total ischemia, as revealed by electronmicroscopy (Brown *et al* 1970) and by biochemical studies (Ahonen *et al* 1970). Further, the presence of a reduced intestinal oxygen consumption and depression of mucosal oxidative phosphorylation and nucleotide synthesis (Bounous *et al* 1963, 1964) indicate that hemorrhagic shock produce hypoxia in the intestinal mucosa.

It would seem most likely *a priori* that hypoxia develops in the intestinal villi during shock as a result of a reduced volume flow of blood. However an indicator dilution technique allowing selective blood flow measurements in the villi (Biber *et al* 1973) suggests that villous plasma flow is not substantially reduced in a 10—20 min experimental period neither during a 30—40 mm Hg hypotension (Lundgren and Svanvik 1973) nor during intense vasoconstrictor fibre activation (8 Hz, Svanvik 1973). Furthermore, mucosal lesions develop during a 2 h hemorrhagic hypotension despite the fact that total intestinal blood flow has returned to about prehypotensive control well before the end of the hypotensive period (Haglund and Lundgren 1973).

On the other hand the indicator-dilution technique clearly demonstrated that *mean transit time* in the hairpin vascular loops of the villi increased from 4—6 s during control to 20—30 s during hypotension. This lowered linear rate of flow would greatly increase the chances of extravascular shortcircuiting of oxygen at the bases of the villi by cross-diffusion, a phenomenon that occurs to a certain extent already at normal blood flows (Kampp *et al* 1968). The mucosa and particularly the tips of the villi may thus become hypoxic in the face of a largely unchanged volume blood flow to the villi due to the presence of the mucosal countercurrent exchanger. The importance of such a mechanism during impaired intestinal blood flow was originally suggested by Lundgren (1967) and was discussed at some length by Chiu *et al* (1970 a) and by Haglund and Lundgren (1973) with regard to the situation during hypotension.

It should in this connection be pointed out that the amount of oxygen supplied by the intraluminal perfusion which prevented the development of mucosal ulcerations is very small. Even if all the oxygen of the intraluminal perfusate were extracted while passing the segment each minute this would not correspond to more than the total amount of oxygen contained in an extra supply of 0.3—0.4 ml fully saturated blood per minute. This observation illustrates the low oxygen demand of the villi, a tissue region which has a high capacity for anaerobic glycolysis, but yet how vulnerable it seems to be to hypoxia.

Filling the intestinal lumen with 5.5 % glucose prior to the hypotension did not reduce the mucosal lesions as compared to control segments or segments filled with saline (Table IV). Chiu *et al* (1970 b) on the other hand reported a protective effect of intraluminal glucose in experiments where intestinal ischemia was induced for 1 h by complete arterial obstruction. An increase of the intestinal lactic acid concentration was observed by Chiu *et al* and it was proposed that the protective effect of glucose was due to its use as substrate for anaerobic glycolysis. Also in the present study an increased intestinal lactic acid production was found (Table II).

although glucose was not able to prevent the ulcerations However in the present experiments the lactic acid production was still high 20 min after relasing the clamp The discrepancy between the results reported by Chiu *et al* and the present study is difficult to explain It may be related to the fact that Chiu *et al* performed their experiments during total intestinal ischemia of the dog small intestine which makes a strict comparison difficult

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From the Department of Physiology University of Goteborg Sweden

Mucosal Hemodynamics in the Small Intestine of the Cat during Reduced Perfusion Pressure

By

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Abstract

Lundgren, O and J Svanvik *Mucosal hemodynamics in the small intestine of the cat during reduced perfusion pressure* Acta physiol scand 1973 *88* 551—563

This study was performed with an indicator-dilution technique that allowed quantitative and separate investigation on the cat small intestine of the mucosal as well as of the villous intravascular flows and volumes When reducing arterial inflow pressure from about 100 to about 30 mm Hg villous plasma volume and mean transit time increased while villous plasma flow remained largely unaltered Concomitantly total intestinal blood flow decreased significantly indicating that a larger fraction of total plasma flow was diverted to the villi at low inflow pressure When intestinal blood flow was reduced by increasing venous outflow pressure villous hemodynamics was largely unaffected Mucosal red cell and plasma flows were affected in the direction of and generally speaking in proportion to total intestinal blood flow These results suggest that the autoregulatory capacity of the villous vessels is larger than that of the vessels in the deeper parts of the mucosa.

The influence of reduced arterial inflow pressure and increased venous outflow pressure on the small intestinal vascular bed has been studied by several authors during the two last decades (see *e g* Selkurt 1955 Haddy and Gilbert 1956 Johnson 1960 1964 1967 Texter *et al* 1962 Hinshaw 1963 Lutz 1966) In these experiments it has repeatedly been demonstrated that the intestinal vascular bed exhibits autoregulation of blood flow *i e* flow tends to remain constant despite variations in arterial perfusion pressure Moreover an increase of venous outflow pressure causes a more pronounced reduction of intestinal blood flow than expected from the decreased perfusion pressure probably due to a constriction mainly of the pre capillary resistance vessels (venous-arteriolar response Johnson 1968) Most authors (*e g* Johnson 1964 Lutz 1966) ascribe these observations mainly to an inherent quality of the vascular smooth muscles according to which an increased (decreased) transmural pressure will *per se* cause vasoconstriction (vasodilatation)

All the abovementioned studies were performed on the whole intestine and its mesentery and due to a lack of method nothing is known about differences in the autoregulatory capacity between *e g* mucosal and muscularis vessels During the

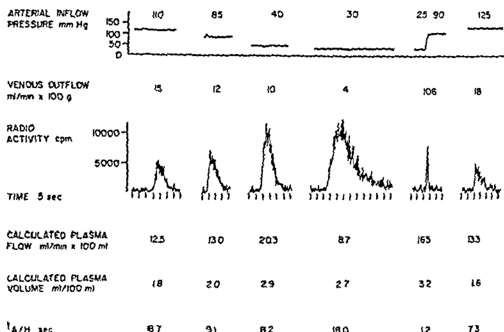


Fig 1 The effect varying arterial inflow pressure (upper panel) on total intestinal blood flow (venous outflow) and on the villous plasma flow plasma volume and mean transit time ($\bar{t}_{A/H}$) calculated from the indicator dilution curves illustrated in the middle part of the Fig For details see text

past years an indicator dilution technique has been developed in this laboratory for quantitative and separate study of mucosal hemodynamics of the cat small intestine (Biber *et al* 1973 a) Below are reported experiments with this technique in which perfusion pressure was decreased by lowering arterial inflow or increasing venous outflow pressure

Methods

The experiments were performed on 2? cats anesthetized i v with chloralose (50—70 mg/kg b w) deprived of food for at least 24 h and without obvious signs of intestinal infection The experimental approach and the operative procedures of the present study were identical to those described fully in an earlier paper (Biber *et al* 1973 a) which also gives full details regarding calculation of regional blood flows and volumes

To summarize the method involves a continuous registration of venous outflow from an isolated intestinal segment *in situ* by means of an optical drop recorder unit The transit of i a injected labelled red cells (^{32}P) or plasma colloids (^{32}P or ^{198}Au) was monitored by a detector in the lumen of the gut Knowing the total tracer amount injected and the total blood flow it is possible to estimate mucosal plasma or red cell flows from the height of the recorded indicator-dilution curve (Biber *et al* 1973 a) Furthermore regional plasma or red cell volumes can be determined from the area under the recorded curve Since the volume of the region monitored by the detector depends on the energy level of the β radiation ^{32}P labelled red cells and plasma colloids were monitored predominantly from the mucosa while ^{198}Au labelled plasma particles were registered only from the villi Arterial inflow pressure to the intestinal segment was registered from a small branch of the intact superior mesenteric artery and could be reduced by an adjustable clamp around the superior mesenteric artery Inflow pressure was varied in steps of 20—30 mm Hg and after 1—4 min of stabilisation at every pressure level tracer injections were made

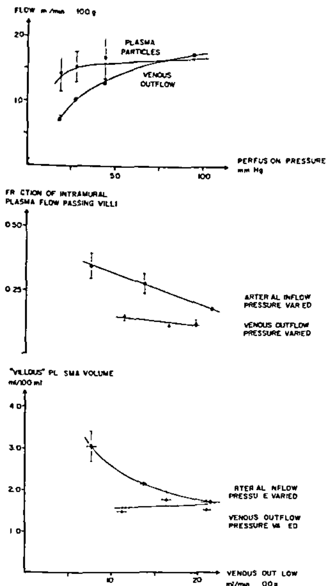


Fig 2 *Upper panel* Cumulated data (10 expts 84 injections) on the effect of varying perfus on pressure on total nte tinal blood flow (enous outflow) and on illous plasma flow (plasma particles) as determined w th [19] Au labelled collo ds Perfusion pressure was varied by partial occlusion of the superior mesenteric artery by means of a clamp Mean arterial hematocrit determined from the experiments of each point varied between 33 and 35 per cent Lines drawn by inspection Bars indicate ± S E

Middl panel The fraction of intramural plasma flow pas ng villi at arious intestinal blood flows Venous outflow was varied either by reducing arterial inflow pressure 10 expts 84 injections) or b nc ea ing venous outflow p es ure (5 expts 53 inject ons) The curve was constructed from the "villous flow values hown in the upper panel of th s F g and in the left panel of Fig 4 assuming a homogeneou blood flow n 0 7 mm long illi Lines drawn by inspection Bars nd cate ± S E

Lower panel Th elation hip betw een total intestinal blood flow and " illous volume determined w th [1] Au labelled colloid part cles The curves are based on the experiments as n the middle panel I

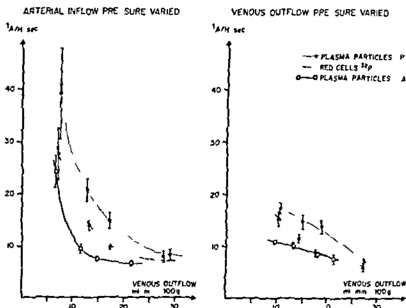


Fig 3 The correlation between total intestinal blood flow and mean transit time ($t_{A/H}$) for red cells labelled with ^{32}P (7 expts 48 injections) and plasma particles labelled with ^{32}P (7 expts 49 injections) and ^{198}Au (10 expts 84 injections) Flow was varied by reducing arterial inflow pressure (left panel) or by increasing venous outflow pressure (right panel) The ^{198}Au curves were based on the same experiments as in Fig 2 and the ^{32}P experiments were identical to those of Fig 5 Lines drawn by inspection Bars indicate ± S E

Venous outflow pressure could be set any desired level by adjustments of the end of the tube draining the drop recorder Tracer injections were made at 4 different venous outflow pressures i e 6 5 13 26 and 33 cm H_2O

Results

A ^{198}Au labelled colloid plasma particles

^{198}Au labelled plasma particles were monitored at their passage through the hairpin loops of the villi (Biber *et al* 1973 a) Parameters measured with this tracer are therefore designed villous

during rest and reduced arterial inflow pressure A typical experiment is shown in Fig 1 in which 0 025 ml of the tracer solution was injected at various levels of arterial blood pressure It can be seen that there was an increase of the height of the recorded indicator dilution curves when inflow pressure was reduced indicating that a comparatively larger fraction of intestinal plasma flow was distributed to the villi During low pressure perfusion the calculated villous plasma volume (determined from the area under the curve) was increased while calculated villous plasma flow (determined from the height of curve) was reduced only at the lowest perfusion pressure Mean transit time ($t_{A/H}$) was significantly increased at the

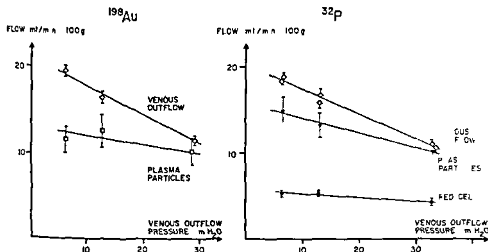


Fig 4 *Left panel* The effect of venous outflow pressure on total intestinal blood flow (venous outflow) and on villous plasma flow (plasma particles) The curves are based on 5 expts (53 injections) in which 3—4 injections were made at each venous pressure level Total intestinal flow resistance increased from an average value of 4 60 to 6 45 PRU_{100} as venous pressure was raised from 6 5 to 30 cm H_2O Lines drawn by inspection Bars indicate ± S E

Right panel The effect of venous outflow pressure on total intestinal blood flow and mucosal red cell and plasma flows Experiments were performed on 6 cats (51 injections of each labelled solution) in which 2—3 injections were made at each venous pressure level Total intestinal flow resistance increased from an average value of 4 70 to 6 10 PRU_{100} as venous pressure was raised from 6 to above 30 cm H_2O Lines drawn by inspection Bars indicate ± S E

lowest pressure level Upon releasing the arterial clamp a transient hyperemia was observed before return to control values

Accumulated data on villous plasma flow during low perfusion pressure from 10 cats are shown in Fig 2 upper panel Total intestinal blood flow showed a certain autoregulation while villous plasma flow was autoregulating to such an extent that the flow was here very little decreased when perfusion pressure was reduced from around 100 to about 20 mm Hg Since total venous outflow was simultaneously decreased to approximately half the value recorded at normal perfusion pressure an increased fraction of total intestinal plasma flow must have been diverted to the villi (*cf* Fig 1) This fraction can be estimated if the length of the vascularized part of the villi is approximately known (*cf* Biber *et al* 1973 b) In the middle panel of Fig 2 this length of the villi was set to 0 7 mm This Fig clearly shows that when the intestinal venous outflow was reduced by lowering arterial inflow pressure an increased fraction of intestinal plasma flow was distributed to the villi

The lower panel of Fig 2 illustrates the accumulated data on the relationship between villous plasma volume and intestinal venous outflow The upper curve of this panel shows that the villous plasma volume was almost doubled venous outflow was reduced by lowering arterial inflow pressure

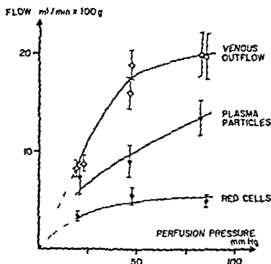


Fig 5 The effect of varying prefusion pressure on total intestinal blood flow (venous outflow) and on mucosal plasma flow (plasma particles) and red cell flow (red cells) Perfusion pressure was altered by occlusion of the superior mesenteric artery by means of a clamp The curves are based on 6 expts in which labelled red cells (43 injections) and labelled plasma particles (41 injections) were alternatingly injected Mean arterial hematocrit determined from the experiments of each point amounted to 33—34 pr cent. Lines drawn by inspection. Bars indicate ± S E

Mean transit time for plasma through the villous hairpin loops was measured with the ^{198}Au plasma particles The correlation between total venous outflow and mean transit time ($\bar{t}_{A/B}$) for villous plasma flow when arterial pressure is progressively reduced is shown on Fig 3 left panel A marked decrease of linear flow velocity through the villous loops appears to occur as venous outflow was lowered by partial occlusion of the supplying artery

In another series of experiments venous outflow pressure was changed instead by adjusting the height of the tube draining the drop recorder The effect of this procedure on "villous plasma flow is demonstrated in the left panel of Fig 4 where flow is plotted against venous outflow pressure A pronounced reduction of intestinal venous outflow was observed when the venous outflow pressure was raised peripheral blood flow resistance increasing from 4 60 PRU_{100} units at a venous outflow pressure of 6 5 cm H_2O to 6 45 PRU_{100} at a mean venous pressure of 28 8 cm H_2O The villous plasma flow seemed however to be less reduced than total flow of the intestinal segment Thus the fraction of total plasma flow passing villi increased slightly when total venous outflow was reduced by increasing venous outflow pressure as illustrated on the middle panel of Fig 2

The effects of an increased venous pressure on villous plasma volume and mean transit time in the villous vascular loops are shown in the lower panel of Fig 2 and on Fig 3 respectively None of the studied parameters were drastically altered as intestinal blood flow was decreased by a raised venous outflow pressure

B ^{32}P labelled red cells and plasma particles

^{32}P labelled blood particles were monitored at their passage through the entire mucosa and to a minor extent also through the submucosa and the muscularis (Biber *et al* 1973 a) Parameters measured with this tracer are therefore designed mucosal

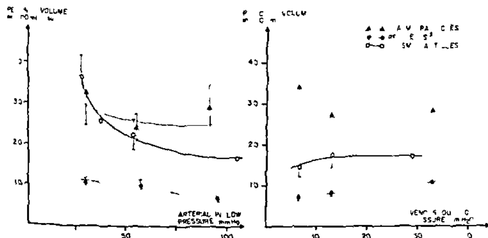


Fig 6 The effect of arterial inflow pressure (left panel) and venous outflow pressure (right panel) on "villous and mucosal intra ascular volumes The experiments in which villous plasma volume (plasma particles ^{198}Au) were determined were identical to those of Fig ? The results with ^{32}P labelled blood (muco al volumes) are based on the e periments in the right panel of Fig 4 and in Fig 5 Lines drawn by inspection Bars indicate ± S E

The effects of a reduced arterial perfusion pressure on mucosal red cell and plasma flows are illustrated in Fig 5 The observations were recorded in 6 expts in which red cells and plasma particles were alternatingly injected The diagram shows that the reactions of the mucosal vessels are in contrast to what is seen in the villous vessels (Fig 2) qualitatively similar to those observed for total venous outflow Moreover the sum of red cell and plasma flows approximates total intestinal blood flow

The right panel of Fig 4 illustrates the effects of an increased venous outflow pressure on mucosal red cell and plasma flows The mucosal flows seem to be reduced to a smaller extent than total venous outflow as was also the case with villous plasma flow (left panel)

The changes in mean transit time for ^{3}P labelled red cells and plasma particles induced by altering arterial inflow or venous outflow pressure are shown in Fig 3 Red cells move faster through the tissue than plasma as expected from the laminar flow of blood The ^{3}P labelled blood particles responded qualitatively in a similar fashion to ^{198}Au labelled colloids upon changes in arterial and venous pressures However comparing ^{3}P and ^{198}Au labelled plasma it is clear that $\bar{t}_{VR}$ for the ^{2}P labelled particles was considerably greater probably due to the larger tissue region monitored with the latter tracer

The left panel of Fig 6 demonstrates that the mucosal content of red cells and plasma increased when reducing arterial inflow pressure but not as markedly as the case with villous plasma volume which is shown also here (*cf* Fig

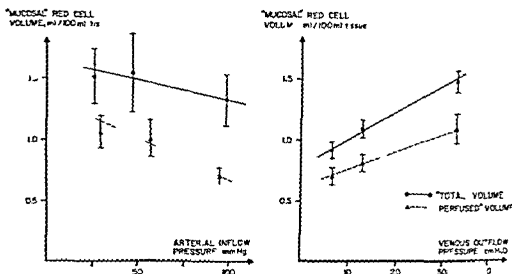


Fig 7 The effect of arterial inflow pressure and venous outflow pressure on "total" and "perfused" red cell volume of the mucosa. Lines drawn by inspection. Bars indicate ± S.E. For details see text.

comparison In the right panel of Fig 6 the effects of raising venous pressure on regional intravascular volumes are shown "Mucosal" plasma volume was somewhat decreased while "mucosal" red cell content was slightly increased upon increasing venous pressure *For comparison the changes in "villous" plasma volume is also* shown.

The "mucosal" red cell content was estimated in two different ways as described in detail by Biber *et al* (1973 a) Usually it was determined from the area under the indicator-dilution curve This volume is named "perfused" volume since it is believed that it measures the intravascular volume in those vessels only that are actually traversed by the injected slug (Biber *et al* 1973 a) "Mucosal" red cell volume was also determined with the "equilibration technique" involving an equilibration of the injected tracer for about 10 min within the circulating blood volume This volume is called "total" volume since virtually all vessels including those that are intermittently closed off from the flow have probably become labelled here. The effect of changes in arterial and venous pressures on these two red cell volumes is illustrated in Fig 7 The "perfused mucosal" red cell volume was considerably affected by both procedures *increasing markedly e g* when arterial pressure was reduced while "total mucosal" red cell volume was only slightly changed in connection with the arterial pressure reduction It should be noted that venous outflow pressure was kept around 13 cm H_2O when the lowering of arterial inflow pressure was performed (Fig 7 left panel)

Discussion

The present investigation concerning the vascular bed of c ı ɹ intı t nt carried out to analyze selectively the reactions of the villou v e stu v ^{198}Au labelled plasma) as compared with those of the *entire* ı (ı m sa vessels studied with ^{32}P labelled blood) upon changes in perfu ı results will be discussed below with respect to their implication c resistance vessels the capacitance vessels and the exchange ve c entities Since it is generally assumed that the hemodynamic effect ı the intestine in connection with changes in perfusion pressure are secon inherent properties of the vascular smooth muscles as influenced by bo h p and chemical environment the results will be discussed along these general lines

The *resistance function* of the mucosal vessels is reflected in the regional bl flow measured with ^{32}P labelled or ^{198}Au labelled blood particles A comparison between the results obtained with the two tracers during reduction of arterial inflow pressure (Fig 2 and 5) suggests that there are considerable *regional* differences in the autoregulatory ability in the superficial and deeper mucosal vessels Thus villous blood flow is almost unaffected by an arterial pressure reduction down to 30 mm Hg, while mucosal blood flow is considerably more reduced upon pressure reduction largely in parallel with the reduction in total blood flow through the intestine This indicates that the vessels supplying the villi have an extraordinary capacity for autoregulation while blood flow to deeper mucosal sections is considerably more influenced by changes in arterial pressure Thus along with a reduction in arterial pressure blood flow is increasingly redistributed from deeper parts of the mucosa to the villous circulation (Fig 2 middle panel)

It might be argued that a change in the extent of mucosal plasma skimming (Jodal and Lundgren 1970) could explain these findings since only villous *plasma* flow was measured It has however been shown that reduction of the arterial pressure if anything raises the normally low villous hematocrit (Jodal and Lundgren 1970)

The observations described above implies that the villi and the deeper mucosal regions are supplied by separate sets of vessels whose smooth muscle cells exhibit different degrees of autoregulation Such an interpretation of the data agrees well with morphological studies (Heller 1872 Mall 1888 Patzelt 1936) which indicate that separate ets of vessels supply the villi and the tissue surrounding the crypts

The reactions of the different sets of intestinal resistance vessels were also studied during raised venous outflow pressure (Fig 4) When this was increased the total intestinal blood flow was reduced more than could be accounted for by the lowered perfusion pressure This observation has been described earlier by several authors and has been named the venous arteriolar response (Johnson 1964) and presumably reflects a myogenic reaction to the rise in transmural pressure As regards this re ponse Fig 4 shows that the flow in the mucosal and particularly in the villous vessels is less reduced than total intestinal blood flow suggesting that venous arteriolar myogenic response is particularly pronounced in m

intestinal vascular bed other than those supplying the mucosa The absence of qualitative difference between mucosal and villous plasma flow in this resp should be contrasted to the more pronounced villous autoregulation when flow reduced by a lowering of *arterial* pressure This suggests that the autoregulation the villous vessels may be relatively more dominated by local chemical influen than by myogenic ones while the reverse seems to be true for some more deep situated vascular regions Had only the myogenic hypothesis of autoregulation be taken into account, one would have expected a relatively more pronounced redu tion of villous plasma flow when transmural pressure was raised from the venc side In part, however, these relative differences in autoregulatory characterist between villous vessels and more deeply situated ones may simply be due to relatively lower pre/postcapillary resistance ratio in the villous vascular circu which would here have the consequence that a rise in venous pressure would le strongly affect the autoregulating precapillary vessels This would then imply th mean hydrostatic pressure in the villous capillaries would be higher than e in the capillaries in the crypts a conclusion which is also suggested by some observa tions discussed below It should however in this connection be pointed out that the observations illustrated in the left panel of Fig 4 might at least in part be due to a methodological error in case the increased venous pressure leads to an erection of the villi towards the intraluminal radiation detector

The *capacitans function of the mucosal vessels* reflected in the calculated intra vascular volumes was determined by the slug injection method (^{32}P labelled blood particles and ^{198}Au labelled colloids) and by the equilibration method (^{32}P labelled red cells)

From the experiments using ^{198}Au plasma particles it is evident that villous plasma content increases when arterial pressure is decreased (lower panel of Fig 7 and left panel of Fig 6) while villous plasma content remains largely unchanged when the intestinal blood flow was reduced to the same extent by a venous pressure rise (Fig 2)

The mucosal plasma volume on the other hand increased only slightly during arterial hypotension (Fig 6 left panel) Thus the increase of mucosal blood con tent in connection with reduction in arterial pressure seems to be located almost exclusively within the villi suggesting an opening up of the capillary networks which this time contain the main bulk of blood volume (see below) The reactions of these parts of the villous vascular bed, which are preferentially responsible for the regional capacitance function thus respond as the resistance vessels proper

Venous pressure elevations cause relatively small alterations in *villous* and mucosal plasma volumes (Fig 6), while mucosal red cell volume (total and perfused) increased 30—60 per cent (Fig 6 and 7) Since the villi probably are normally perfused by blood of low hematocrit due to a plasma skimming mechanism (Jodal and Lundgren 1970) the mucosal red cell volume is predominantly located in the deeper mucosal layers An increase of mucosal red cell content is therefore probably caused by a distension of the small veins in this intestinal region

In previous reports (Wallentin 1966 Haglund and Lundgren 1972) the effects of arterial inflow and venous outflow pressure on the total intestinal blood volume was studied by plethysmography Lowering arterial inflow pressure induced only a small decrease in intestinal blood volume probably due to the fact that the pre capillary autoregulatory adjustments keep a fairly constant mean capillary pressure and hence, a fairly constant mean distending pressure also within the postcapillary capacitance section (Haglund and Lundgren 1972) Comparing these observations with those illustrated in Fig 6 a marked difference is noted particularly as regards the villous plasma volume The explanation for this discrepancy is probably that the villous plasma volume is contained almost exclusively within capillaries a vascular section which in most other tissues contributes with only a minor fraction to the regional blood volume A doubling of the villous capillary blood volume will therefore very little influence the blood volume of the intestine as a whole since this is mainly contained within the voluminous postcapillary section

Raising venous outflow pressure causes abrupt increases of total intestinal blood volume, of the same order of magnitude as that reported above for mucosal red cell volume These changes are as discussed above probably induced by a distension of the intestinal postcapillary compartment

The precapillary sphincter function of the villous vascular bed controlling the *surface area available for exchange but also forming part of the precapillary* resistance vessels is reflected in the villous plasma volume since the major part of the villous vascular bed is made up by the capillary network (Biber *et al* 1972 a) Lowering arterial inflow pressure markedly relax the sphincters along with the resistance vessels increasing villous capillary volume and hence area almost twofold (Fig 2 lower panel) while elevation of venous outflow pressure has no significant effect on villous plasma volume (Fig 2 and 6)

Using a plethysmographic technique Haglund and Lundgren (1972) showed that the intestinal capillary filtration coefficient (CFC) being a semiquantitative measure of *total* capillary pore area of the small intestine is rapidly increased about 50 per cent above control upon lowering arterial pressure to 30 mm Hg Further more Johnson and Hanson (1966) showed in the dog intestine that increasing venous outflow pressure about 20 cm H_2O decreased CFC to about 30 % of control A comparison between these two sets of data suggests that changes in *total* intestinal capillary surface area is not necessarily accompanied by corresponding alterations in villous capillary area This may imply that an extensive capillary section in the intestinal wall other than the villous one greatly influences the CFC determina tions It is proposed that this capillary section is located around the crypts which form a very well vascularized region (Lundgren 1967) An extensive porosity in these vessels would also result in a reduced colloid osmotic pressure difference and hence call for a lower mean capillary hydrostatic pressure to maintain a Starling equilibrium A comparatively large pre to postcapillary resistance ratio during resting' equilibrium in the crypt vessels may account for such a relatively low capillary pressure as discussed above

The difference between perfused and 'total' red cell volume (Fig 7) represents an indirect measure of precapillary sphincter activity As discussed above this difference is probably represented by such vessels, mainly capillaries and venules which are not perfused at the moment of determining red cell volume with the 'slug injection' method The conclusions drawn from Fig 7 are similar to those of Fig 6 *i e* relaxation of the sphincters occur readily upon reduction of arterial pressure while pressure increases cause only slight changes

Linear flow rates in the mucosal and villous vessels can be estimated from the measurement of the mean transit time ($\bar{t}_{A/H}$) for red cells and plasma particles (Fig 3) The transit of ^{198}Au particles in the villous capillaries is markedly retarded when total intestinal blood flow *is reduced to less than 10—12 ml/min × 100 g* as caused by a lowering of arterial inflow pressure to less than 40—50 mm Hg (see Fig 3, left panel) This is explained by the earlier described relaxation of the precapillary resistance sphincter sections of the villous vascular circuit, thus increasing its plasma *volume* concomitantly to a largely unaltered villous plasma flow The linear velocity of plasma particles in the villous capillaries then becomes less than 0 07 mm/s if the villus is assumed to be 0 7 mm long

This is indeed a very low linear rate of flow along the villous vascular hairpin loops and it must be of particular functional importance with respect to the existence of a countercurrent exchanger in the intestinal villi (Lundgren 1967) Such low linear rates of flow will make the exchanger very efficient in hindering net transport to or from the villi by means of the blood stream Thus the absorption of easily diffusible solutes will be markedly retarded Furthermore solutes delivered to the villi *via* blood may during hypotension be increasingly short circuited extravascularly at the villous base Thus oxygen may become almost entirely excluded from the villous tips in *e g* shock situations explaining the intestinal mucosal lesions seen in shock in dog cat and even man (for a discussion see *Haglund Lundgren and* Svanvik 1972 Haglund and Lundgren 1973)

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Acta physiol scand 1973 88 564—570
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Effect of Training with Eccentric Muscle Contractions on Skeletal Muscle Metabolites

By

FLEMMING BONDE PETERSEN,[1] JAN HENRIKSSON and HOWARD G KNUTTGEN

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Abstract

BONDE PETERSEN, F J HENRIKSSON and H G KNUTTGEN *Effect of training with eccentric muscle contractions on skeletal muscle metabolites* Acta physiol scand 1973 88 564—570

The effect of training with eccentric contractions on skeletal muscle metabolism was investigated with human subjects engaged in cycling exercise Experiments involving extended (30 min) exercise at high relative intensities were performed before and after a 5 week period of training (25—30 min per day 22 days) Muscle biopsies (lateral portion of m quadriceps femoris) were taken at rest after 4 min exercise and termination of exercise Expired air was collected periodically for determination of respiratory exchange $\dot{V}_{O_2}$ and heart rate were lowered by training (e g after 26 min of exercise from an average of 0 96 to 0 74 l×min⁻¹ and 115 to 96 beats×min⁻¹ respectively) Muscle metabolite concentration evidenced no significant changes but respiratory exchange ratio (R) was decreased (after 10 and 26 min from an average of 0 85 to 0.80 in both instances) Metabolic determinations offered no explanation for the lowered O_2 cost of exercise nor evidence for or against the hypothesis that the muscles utilize the mechanical energy received during eccentric exercise

The effect of training on the oxygen cost of eccentric exercise (or negative work) has been investigated (Klausen and Knuttgen 1971) It was observed that training in extended exercise periods with eccentric contractions both lowered the oxygen cost of such exercise and decreased the tendency toward significant increments in oxygen uptake ($\dot{V}_{O_2}$) during the course of a single exercise period

The possibility was suggested that the energy received by the muscles during eccentric exercise could be employed to spare the biochemical energy release processes and that the lowering of $\dot{V}_{O_2}$ during training represented an adaptation of the muscle cells to better utilize the energy in this manner This hypothesis was tested in short term experiments by Bonde Petersen *et al* (1973) On the basis of studies

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Key words Exercise Muscle biopsies Oxygen uptake

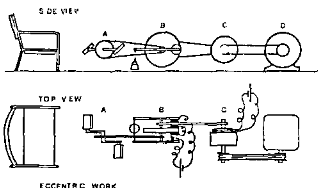


Fig 1 Side and top view of the applied ergometer A is the cycle crank B the fly wheel of the Krogh cycle ergometer with magnets counter balanced by the load C is an induction clutch consisting of two freely rotating parts The part which is connected to the fly wheel of the cycle ergometer by a chain is furnished by electromagnets activated from a variable power supply When the motor D is rotating the other part of the clutch by means of two wedge rubber bands the induction forces will create a torque which is transmitted from C to B This torque is varied during calibration until a known torque on the magnets of B and a desired pedal rate of the unloaded crank A is obtained During the experiments the electromagnets of B are switched off and the direction of the pedal revolutions is reversed The subject will then resist a known torque in pace with a metronome

of muscle metabolites obtained by a biopsy needle technique no evidence was found to either confirm or deny the hypothesis

A difference between the metabolism during concentric and eccentric exercise might however appear as a result of a period of eccentric training The purpose of the present study was therefore to investigate the effect of training with extended bouts of exercise involving eccentric muscle contractions upon skeletal muscle metabolism as assessed by the concentration of certain muscle metabolites

Methods

6 healthy male subjects were employed in this study Personal data are presented in Table I The method of exercise was cycling (60 rpm) in a sitting position (Nielsen 1938) on an ergometer employing an induction clutch (Bonde Petersen 1969) as shown in Fig 1

Maximal $\dot{V}_{O_2}$ was determined for each subject in pre tests on a conventional bicycle ergometer (Elema) with the subject employing concentric contractions Each subject also performed a

Table I Personal data of subjects and work intensities employed in testing and training with eccentric exercise

Subject	Age	Height cm	Weight kg	$\dot{V}_{O_2}$ max l/min	Eccentric exercise intensity Watts
1	18	180	75 5	3 18	155
2	19	181	66 5	3 09	210
3	21	175	65 8	3 33	185
4	[illegible]0	172	59 9	3 18	175
5	[illegible]0	189	76 0	3 53	2[illegible]5
6	19	18[illegible]	[illegible]9 8	4 23	[illegible]60

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TABLE III Mean values of the concentrations of ATP CP glycogen and lactate in the lateral portion of the m. quadriceps femoris (mmoles × kg wet muscle) and lactate concentration in whole blood (mmoles × l⁻¹) in the pre training s[illegible] pre and in the p[illegible] training (post) after 5 weeks with eccentric exercise Th[illegible] alues r pres[illegible] res[illegible] and exercise at the 4th and the 30th min

		Rest		Exercise			
				4 min		3[illegible]	
		pre	post	pre	post	p[illegible]	[illegible]
ATP							
	$\bar{x}$	3 90	4 23	4 20	4 38	4[illegible]	3[illegible]
	SE	0 35	0 25	0 20	0 13	0[illegible]	[illegible]
CP							
	$\bar{x}$	17 00	16 97	16 03	16 27	14 [illegible]3	4[illegible]
	SE	1 48	0 67	0 84	0 70	0 6[illegible]	0 83
Glycogen							
	$\bar{x}$	79 0	71 7	81 5	79 3	68	68 4
	SE	6 5	4 4	7.2	4 3	2[illegible]	5 9
Lactate in muscle							
	$\bar{x}$	1 03	0 88	1 07	1 17	1 [illegible]	1 23
	SE	0 07	0 08	0 06	0 12	0 14	0 12
Lactate in blood							
	$\bar{x}$	1 53	0 97	1 57	1 03	1 33	0 83
	SE	0 15	0 12	0 11	0 14	0 14	0 09

Results

Table II presents the results for the oxygen uptake (V_{O_2}) respiratory exchange ratio (R) ventilatory equivalent ($V_{E_{BTPS}} \times V_{O_2}^{-1}$) and heart rate (HR) during the eccentric test exercise both before and after the training period Due to the training a significant decrease in oxygen uptake ($0 01 > P > 0 001$) was seen at both 10—14 min and 26—30 min during exercise The R did not show any significant difference during the first 4 min of the exercise period whereas there was a decrease in R from 0 85 to 0 80 at 10—14 m[illegible] and 26—30 min This difference in R was significant ($0 01 > P > 0 001$) at 10—[illegible]4 min but showed only a tendency ($0 05 > P > 0 02$) at 26—30 min

Pulmonary ventilation as evidenced by the ventilatory equivalent was quite appropiate to the V_{O_2} both pre and post training

Heart rate decreased as a result of training but the significance for this decrease was only apparent ($0 01 > P$) during the last 20 min of the exercise test (Table II)

The perceived exertion evaluated by the Borg scale decreased in parallel with the decrease in heart rate This decrease was significant ($0 01 > P$) during the last 10 min of the exercise test (Table II)

The results of the biochemical analyses of muscle biopsies and blood samples are presented in Table III The ATP concentration in the lateral portion of the m quadriceps femoris showed no significant changes either during a single experiment or when the pre trained values were compared to the trained values

The CP values decreased during the exercise period both in the pre trained and in the post trained state The pattern for this decrease seemed to be similar in the two states but the P values were above 0 02 for all changes with but one exception This was observed in the difference between the resting values and the 30 min values in the trained state where $0.02 > P > 0.01$

The lactate values in muscle and blood evidenced no definite pattern except for a slight but insignificant increase ($P > 0.02$) in the muscle biopsies at 30 min as compared to the resting values in both conditions A tendency towards a lowering of muscle glycogen stores was observed in both tests but the significance was above 0 02 in both cases

The results for the isometric leg strength and the vertical jump test showed no significant variations ($P > 0.02$) due to the training The results are therefore not presented

As mentioned 2 subjects performed the training exercise at 70 % 2 at 80 % and 2 at 90 % of the intensity corresponding to their maximum V_{O_2} No significant variation in the pattern of reaction to the training period was seen that could be attributed to this variation in exercise intensity All the results are consequently treated together

Discussion

The present results concerning oxygen cost and heart rate are in accordance with the observations of Klausen and Knuttgen (1971) i e that a period of training with eccentric exercise would result in a significant decrease in both parameters during long term eccentric exercise The present experiments do not demonstrate any clear cut evidence as to the mechanism behind the lower O cost of eccentric exercise after training but certain possibilities can be discussed

One possibility is that the training will result in a better utilization of the oxygen as a result of enzymatic adaptation The muscle biopsies were investigated with emphasis on carbohydrate utilization and no significant changes in these metabolites were observed with the present technique If an enzymatic adaptation has taken place with respect to other metabolites cannot be determined from the biopsies

There was a decrease in R as a result of the training This indicates that the training has evidently influenced the ratio between combusted carbohydrates and free fatty acids during eccentric exercise

The present results might indicate that even after a period of eccentric training there is a tendency for the muscles to shift to a form of metabolism where fat is more readily used than during concentric exercise during conditions where similar V_O is elicited If this is so this is not in accordance with the observation in the present experiment that the oxygen cost for the same amount of eccentric exercise has decreased as the consequence of the eccentric training It could be expected that the oxygen cost for eccentric exercise would increase if the muscle metabolism is switched from carbohydrate to fat metabolism as it is generally accepted that the oxygen cost

for a certain muscle work is greater if fat is used when c a d r c mdr
A switch over from carbohydrate to fat metaboli m can et c ot l th c
planation for the decrease in V_{O_2} seen after a period of t n with ecc
exercise

Pulmonary ventilation was consistently appropriate to V_O t
equivalent showed no significant variation as a re ult of the trai I
that the change in respiratory exchange ratio wa not a consequ t m
ventilatory pattern

Heart rate decreased significantly by training and in clo e compar
decrease in V_O indicating a relationship or perhaps dependency The H
central circulation during eccentric exercise appears dominated by th n
demands of the active tissues with other factors such as muscle ten ion l n
minor roles

The rated perceived exertion as evaluated by the Borg scale showed a similar pattern to heart rate and V_O There was a significant lowering in the perceived exertion for the same eccentric exercise intensity as a result of training The reason for this is obscure One possibility is that exertion is perceived from a combination of peripheral impulses elicited in the tendon organs and muscle spindles and central impulses related to such factors as V_O If this is so the decrease in perceived exertion is explained by the concomitant decrease in V_{O_2} due to the training

It is also possible that the peripheral impulses may be changed due to the training either as a consequence of increased thresholds for stimulating the Golgi tendon organs and muscle spindles or as a consequence of changes in visco elastic properties of the trained muscles If the properties of the muscles are changed in the direction of decreased elasticity a passive stretching of the muscles would call for a greater force This could imply that the number of active motor units during eccentric exercise would decrease

In this way it would be possible to explain the changes seen in the present study If the number of active muscle fibres decreased as a result of training the oxygen cost would also decrea e and a decrease in heart rate and perceived exertion would follow At the present time it would seem that an increase in visco elastic stiffness was the most likely reason for the changes observed after the eccentric training

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Acta physiol scand 1973 88 571
From the Department of Toxicology Pharmacia AB

Effects of Hemodilution Induced by Hyperoncotic on Cardiac Contractility

By

KAREL PAVEK

Received 15 November 1972

Abstract

PAVEK K *Effects of hemodilution induced by hyperoncotic dextran 40 on cardiac contractility* Acta physiol scand 1973 *88* 571—576

In 5 pentobarbital anesthetized dogs with autonomous nervous activity blocked with atropine and propranolol 1 g/kg of dextran 40 as 10 % solution was rapidly infused into the right atrium After dextran the heart rate was not changed while the mean arterial pressure increased slightly by 5 mm Hg Cardiac contractility was studied by a continuous display of left ventricular pressure velocity curves. Peak velocity of contractile elements dP/dt/P estimated at comparable enddiastolic pressure was not consistently changed after dextran A higher stroke volume at comparable enddiastolic pressure after dextran suggests improved ventricular function This might be caused by a decreased aortic impedance No signs of depressed cardiac contractility occurred after a large dose of dextran 40

Despite its extensive use in states of distressed circulation little is known about the direct effect of dextran of hyperoncocity and of hemodilution on myocardial contractility Statements in two recent reports suggest a depressed ventricular function following dextran infusion in patients with coronary heart disease (Russel *et al* 1970 Khaja *et al* 1971) These studies are open to the misinterpretation that dextran has a negative inotropic effect However dextran was used in both these investigations for deliberate induction of changes in left ventricular enddiastolic pressure in order to construct the ventricular function curve Thus using dextran infusion as an investigative tool these two studies have demonstrated that some patients operate on the flat portion of the ventricular function curve

A more confusing report has recently concluded that in young blood donors myocardial contractile force is much depressed by dextran (Dahlgren 1971) This conclusion was based on the finding of a decreased amplitude in the first heart tone a hitherto unknown method of quantitative evaluation of myocardial contractile force This finding might be more easily explained by presumed dependence of heart sound on changes in ventricular volume

On the other hand using experimental technics an improved ventricular function curve has been observed after extensive isovolemic blood dextran exchange (Fowler and Holmes 1971) The authors however concluded that this effect was mostly related to decreased afterload

Thus until now no acceptable evidence concerning a direct effect of dextran on heart muscle has been published The present investigation has been directed towards answering the question whether or not the contractile state of the heart muscle and the pumping action of the left ventricle is directly influenced by a rapidly infused large dose of dextran 40

Methodological Approach

Current assessment of cardiac contractility is based to some extent on an analysis of the heart as a pump (the Frank Starling relation) At present, a quantitative understanding of myocardial performance rests upon Sarnoff's concept of the family of Starling curves expressed by a curvilinear relationship between the left ventricular pressure and stroke volume (or stroke work) This relationship demonstrates graphically the level of heart contractility an increase is associated with shifts of the curve upwards (to a higher stroke volume) and to the left (to a lower enddiastolic pressure) a decrease in contractility is associated with shifts in the opposite directions While the concept is basically valid its usefulness is somewhat limited in the intact circulation rapid reflex adjustments affecting the circulation prevent the description of a true ventricular function curve and extracardiac factors such as aortic impedance can markedly alter left ventricular ejection (survey by Braunwald Ross and Sonnenblick 1968) Furthermore Rushmer's group (survey by Rushmer 1970) analyzed ventricular performance in conscious dogs and showed that in a number of situations ventricular contractility is altered more in terms of "rate of change" than in terms of Frank Starling relationship

The heart has also been studied over the last ten years from the point of muscle mechanics in relation to "active state or "force-generating potential of the contractile elements (survey by Braunwald Ross and Sonnenblick 1968) The most fundamental property of contractile elements is force velocity relationship described for skeletal muscle by A B Hill in 193 At maximum developed force velocity of shortening is zero while with zero load a maximum velocity of contraction is reached. Recently these experimental approaches were extended to the intact heart In search for a measure of heart muscle mechanics which is independent of preload and afterloald, an analysis of left ventricular pressure P its derivative in time dP/dt, and velocity of contractile elements V_{CE} calculated as dP/dt/P during the isovolumic part of systole was recommended (Sonnenblick *et al* 19 0 Mason *et al* 19 0 Mason *et al* 19 1 Kravenbuhl *et al* 19 1 Mirsky *et al* 1971) However also this approach has been criticized (Kravenbuhl *et al* 19 1 Pollack 1971 Mirsky 1971 The most recent information available recommends the use of dP/dt P Parmley *et al* 1972 or of developed pressure DP instead of P Grossman Personal communication for analysis of contractility

In order to stabilize heart rate and exclude neurogenically mediated effects the present experiments were performed after the blockade of autonomic nervous system with atropine and propranolol Peak dP dt P or linear extrapolation of velocity towards the enddiastolic pressure were used as an index of relative value to compare velocities before and after dextran infusion at comparable enddiastolic pressure heart rate and mean arterial pressure Simultaneously cardiac output was measured for construction of the ventricular function curve

Material and methods

5 pentobarbital anesthetized Vorsteh dogs weighing 16 – 6 kg were used atropine 1 mg/kg) and propranolol 1 mg kg were used as blocking agents of autonomous nervous system Right ventricular output was measured in triplicate by thermodilution technique Systemic arterial pressure was obtained from the femoral artery using a Statham transducer Following the recommendations of Mirsky 19 1 left ventricular pressure was measured by rapidly responding pressure tip-transducer SF 1 Statham and pressure velocity loops were computed continuously The method described by Grossman 19 1 with further details in personal communication was used to obtain dP/dt/P plotted against P in the form of continuous loops displayed on

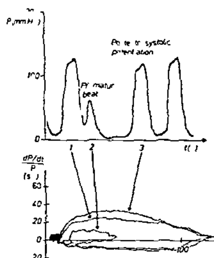


Fig 1

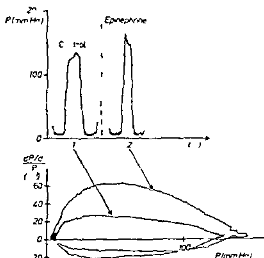


Fig 2

Fig 1 A record of left ventricular pressure with a premature beat is shown in the upper part corresponding pressure velocity loops computed from the ventricular pressure appear in the lower part Note that the peak velocity (dP/dt/P) of the systole following the premature beat is higher (3) than the peak velocity of the normal beat (1) This demonstrates postextrasystolic potentiation of contractility

Fig 2 Effect of 6 μg of epinephrine on the shape of left ventricular pressure curve and the pressure-velocity loop Note that the systole is shortened and the pressure reaches a higher value in a shorter time Consequently the peak velocity is 2 5 times higher

oscilloscope These observations were stored using tape recorder (Tandberg) and later re recorded at a reduced speed on a X Y recorder A mean value of several heart beats was evaluated for each situation A V_{max} at enddiastolic load was also measured In each situation tested changes in V_{max} were similar to changes in peak at dP/dt/P

After completing baseline measurements a solution of dextran 40 (Rheomacrodex® 10 % in saline 1 g/kg of b wt) warmed to body temperature was infused into the right atrium over 3 to 6 min Additional 5 min were allowed for mixing During this period the left ventricular enddiastolic pressure reached its maximum level The above mentioned measurements were repeated three to four times at different enddiastolic pressure step-wise decreases in this pressure was induced by controlled bleeding The successive measurements were separated by a 5 min period

Results

At first sensitivity of the method was tested Fig 1 shows an example of postextra systolic potentiation of contractility Fig 2 demonstrates the marked effect of 6 μg epinephrine on the velocity of CE After atropine and propranolol the loading effect of the same dose of epinephrine is very marked but the effect on contractility is attenuated (Fig 3) Heart rate with an average value of 102 b/min before dextran remained essentially unchanged after dextran with an average value of 106 b/min (pairwise analysis $p > 0.1$) The average value of mean arterial pressure was in creased slightly but significantly from 110 to 115 mm Hg ($p < 0.05$) C

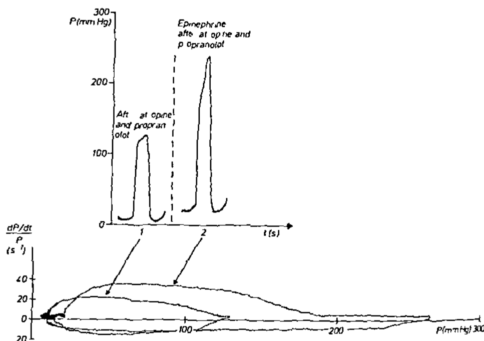


Fig 3 The typical effect of epinephrine shown in Fig 2 is modified by atropine and propranolol While both the afterload caused by intensive peripheral vasoconstriction and the preload caused by increased filling of the ventricle are much increased the peak velocity increased only 1 7 times Thus the contractility increasing effect of epinephrine is substantially attenuated by autonomic blockade

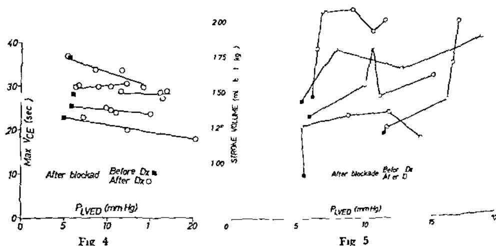


Fig 4

Fig 5

Fig 4 Maximum velocity of contractile elements plotted against the left ventricular end diastolic pressure in 5 dogs Autonomous nervous system was blocked by atropine and propranolol Control values immediately before dextran are shown as squares values after dextran at different enddiastolic pressures are shown as circles A slight dependence of Max VCE on the enddiastolic pressure is apparent The Max VCE *is not modified by dextran*

Fig 5 Stroke volume is plotted against the enddiastolic pressure Squares represent control values while circles represent values after dextran With higher filling pressure a *rise in* stroke volume *is* apparent At a closely similar enddiastolic pressure there is a slightly to moderately increased stroke volume after dextran

put and stroke volume were augmented substantially in each dog The resulting values of dP/dt/P are plotted against enddiastolic pressure on Fig 4 From the data collected it seems that dP/dt/P is slightly dependent on the level of enddiastolic pressure In the range measured there presumably exists a linear relationship between diastolic pressure and peak dP/dt/P In each of the 5 expts dP/dt/P before dextran (black squares) was either lower or at the same level when compared to the linearly extropolated line fitted to values after dextran

A plot of stroke volume at different levels of enddiastolic pressure (Fig 5) reveal a general dependence of stroke volume on the level of pressure It may be observed that at similar enddiastolic pressures slightly to moderately higher stroke volumes appeared after dextran when compared to control values before dextran

Discussion

No sign of depressed myocardial function after a large dose of dextran 40 was observed by either method used

The velocity of contractile elements was evaluated as peak dP/dt/P A decreased peak value at closely similar enddiastolic pressure aortic pressure and heart rate would indicate a decreased cardiac contractility After dextran however the peak velocity was lightly higher in 2 cases and essentially unchanged in 3 cases It is concluded that no consistent changes in peak dP/dt/P occur after dextran 40 The method used meets well Mirsky s criticism (1971) but has at least with the present equipment source of error not mentioned in the literature The computation is based on dP/dt/P = d log P/dt (Grossman 1971) From the equation it appears that a temperature dependent drift of zero of the pressure transducer (SPI Statham) as well as setting of the zero working point of the logarithmic amplifier are influencing the shape of pressure velocity loop since a maximum amplification in the logarithmic amplifier occurs with a pressure approaching zero a drift of zero and setting of the log amplifier are distorting the resulting velocity only through the denominator in dP/dt/P while the first derivative of P is independent of absolute pressure level With increasing left ventricular pressure the error in the denominator becomes negligible thus the pressure velocity loop is distorted mostly during the beginning of isovolemic systole Due to the possible errors mentioned above in our experiments the absolute values of velocities might be in systematic error and slightly dependent on the level of enddiastolic pressure Having this in mind only changes in dP/dt/P related to enddiastolic pressure were evaluated as indicated on Fig 4 This method left no doubt that the peak velocity of contractile elements is not consistently modified by dextran 40

Also the ventricular function curve did not show any unfavorable changes after dextran To prove a depression one would except a lower stroke volume at a comparable enddiastolic pressure In present experiments just opposite was found namely a higher stroke volume at a comparable enddiastolic pressure This finding together with an unchanged heart rate and the same aortic impedance would sugg

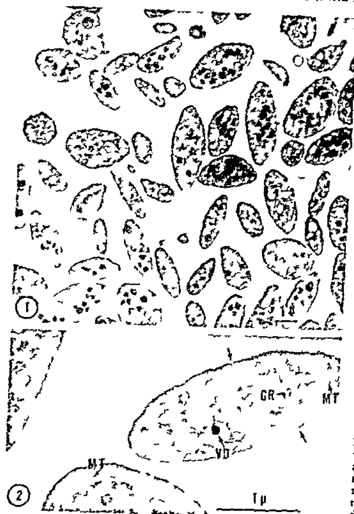


Fig 1 Normal cat platelets with regular and discoid shape ×7500

Fig 2 Normal cat platelet at higher magnification Note the vesicles in the submarginal area Arrows indicate communication with the surrounding medium α granules (GR) microtubules (MT) very dense body (VD) ×30 000

infused without causing any effect in the pulmonary arterial pressure or in the number of circulating platelets

The explanation for the disappearance of the response could be 1) lack of reactivity in the remaining circulating platelets 2) that their number had become *too* low or 3) that the smooth muscles of the lung vessels had become non reactive As to the first possibility the damage of the platelets caused by collagen might be pronounced We have therefore examined the ultrastructure of the circulating platelets from animals which had been exposed to a varying number of collagen infusions

Materials and Methods

Animals 4 cats weighing between 3 5—4 kg were used They were anesthetized by *i p* injections (30 mg per kg b wt) of sodium pent barbitone (Nembutal® Abbott)



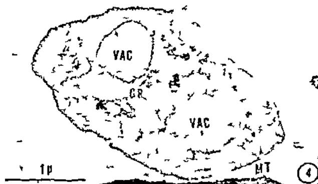


Fig 3 Cat platelets obtained from aortic blood after one injection of collagen Pronounced shape changes with spiny spheres Note the oc currence of vacuoles (VAC) in some of the platelets Pseu dopods (PS) ×7500

Fig 4 A platelet from the same sample as de cribed in Fig 3 The vacuoles (VAC) contain an amorphous sub stance α granule (GR) microtubules (MT) ×30 000

Experimental procedure The animal was artificially entilated and the thorax was op ned Polyethylene catheters were introduced into the pulmonary artery and the left at ium and flow was measured with a Square wave electromagnetic flow meter (Nycotron O lo) the flow probe being placed around the ascending aorta Fu ther experimental details ha e been de scribed in a previous report (Bo and Hognestad 1972)

Platelet counts were carried out with the method of B echer and Cronkite (1950)

Collagen suspension prepared accord ng to Holmsen (1969 vas infused i.v in doses of 0 5 ml each given in the course of a 1 min period When th subsequent pressu e response had vanished, a new response could be obtained as a r sult of a repeated infusion of colla en (0 5 ml)

Blood samples we e obtained from aorta th ough the polyethylene catheter and collected directly into a glass tube containing the fixative Blood samples were drawn before the first collagen infus on after the f rst and second infusion and finally when a collagen infu ion had no more pre u e effect As a control blood was al o collected in some animals after epeated infus ons of 0 5 ml of the uspending medium for ollagen wh h was 0 1 per cent acetic acid

Elect n n oscopy Blood was drawn directl into the fixative 2 5 glutaraldehyde in 0 1 M pho phate buffer 3 5 ml blood to 6 5 ml of the f xative The platel ts were fixed for 1 h and then post fixed for 1 h in osmium tetroxide Dehydration was carried out in graded ethanols and th specimens were embedded in Epon 812 Sect on ng was carried out with a LKB Ultrotome Ult amic otom and the se tions stained with u anyl a tate and lead citrate The sections were examined in a Siemens Elmiskop 1 electron mi oscope



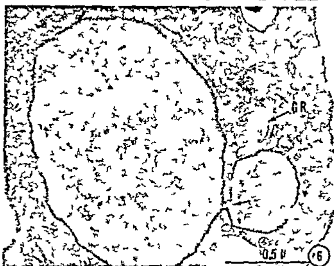


Fig 5 Platelets from aortic blood drawn after repeated injections of collagen which no longer caused any pressure effects on the lungs Pronounced morphological alterations of the platelets with shape changes vacuolization (VAC) and degranulation ×7500

Fig 6 Detail of communicating (arrow) vacuoles containing amorphous material α granules (GR) ×60 000

Results

I Control studies

Cat platelets which were obtained before infusion of collagen, showed a similar ultrastructure to that of platelets from other species They were regular with a discoid shape and only few pseudopods could be noted (Fig 1) The number of dense bodies appeared to be higher than in human platelets but less than in platelets from rabbits (Fig 1) In many of the platelets small vacuoles with a diameter of about 500 Å were noted and at the surface they appeared to be in communication with the surrounding medium (Fig 2) They probably represent the surface-connecting system, but appeared to be more regular in size than those found in human platelets

The platelets obtained after repeated infusions of the acetic acid were similar to those taken before the infusion indicating that the suspending medium for collagen had no effect on the platelet morphology



Fig 7 A platelet with a vacuole appearantly in communication with the surrounding medium (arrows) Dense tubular system (DT) mitochondrium (MIT) microtubules (MT) ×60 000

II Ultrastructure of platelets from animals exposed to collagen

Platelets obtained after the first infusion of the collagen suspension showed shape alterations as they were irregular sphaeric with pseudopods (Fig 3) The cytoplasm varied in electron density and occasional large vacuoles containing an amorphous substance were observed (Fig 4) Usually the internal organelles appeared to be well preserved

In samples taken after the second collagen infusion the findings were similar to those observed after only one injection but the vacuolization was more pronounced

After repeated infusions of collagen when pressure responses could no longer be produced the platelets had undergone pronounced changes Bizarre shape changes occurred but many of the platelets also appeared regular sphaeric (Fig 5)

organelles were no longer preserved and several platelets appeared to be devoid both of *a* granules and of *mitochondria* The *cytoplasm* showed varying election density

The vacuolization of many of the platelets was striking (Fig 5 6 and 7) Occasionally the vacuoles appeared to occupy most of the *platelet* (*Fig* 5) They contained amorphous masses whereas periodic structures indicative of collagen or fibrin were not found The vacuoles appeared to be in contact with each other (Fig 6) but also with the surface (Fig 7)

Discussion

The effects of collagen on blood platelets have previously been studied in *in vitro* experiments (Hovig 1963) Morphological changes with degranulation have then been found both in aggregated platelets and in single platelets In those studies however, the platelets were exposed to a certain amount of collagen for a considerable period of time and under stirring The conditions *in vivo* will differ since the platelets aggregated by collagen are trapped in the lungs and other organs, whereas the remaining circulating platelets are mainly not aggregated Furthermore the conditions in circulating whole blood differ in many ways from those in anticoagulated platelet rich plasma in a tube

The findings of only minor alterations in the platelets after the *first injection* of collagen are therefore not surprising As judged from the morphological appearance such platelets are likely to be viable but activated Even after a second injection of collagen this appears to hold true

After repeated injections of collagen however when no more pressure response followed the collagen stimulus the platelets were markedly changed The changes must be related to collagen as the suspending medium exerted no effect on the platelets As many of the collagen stimulated platelets appeared devoid of organelles indicating release of platelet components they are probably no more able to react This damage might well have been induced by a contact with platelet aggregates in the pulmonary circulation *It seems likely therefore that the lack* of effect of collagen on the pulmonary vascular resistance after repeated injections is related to the deficient platelets In addition the reduced number of circulating *platelets* and a possible reduction in smooth muscle reactivity may still play a role

The vacuolization of the platelets was a striking observation The vacuoles contained amorphous material but there was no evidence of phagocytized collagen It is therefore not likely that the vacuoles reflect a phagocytic process It seems more reasonable that they represent a sign of degeneration of the platelets The channel from a vacuole to the platelet surface may indicate that the content of the vacuole is being discharged to the surroundings and that it thus reflects a stage of the release reaction

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Acta physiol scand 1973 88 584—586
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Effect of Intrapulpal Pressure Stimulation on the Activity of Sensory Nerves of Dental Pulp

By

MARTTI O K HAKUMÄKI and MATTI V O NÄRHI

An increase in the afferent pulpal nerve impulses of the canine tooth elicited by intrapulpal pressure elevation was observed in cats A positive correlation was found between the pressure impulse of the pulpal pressure and the number of afferent intrapulpal impulses

It has been shown in cats that the elevation or lowering of intrapulpal temperature elicits an increase in the nerve activity of intrapulpal origin (Anderson Hannam and Matthews 1970) Some of the receptors in dogs are sensitive to hyper or hypotonic fluids (Yamada 1963) Van Hassel (1971) has observed in man that the intra pulpal pressure is about 25 mm Hg and in pulpal inflammations this pressure rises in monkeys from the normal value to 50—60 mm Hg (Stenvik Iversen and Mjör 1972) Rhythmic bursts of impulses associated with heart beats has also been observed in pulpal afferent nerves in dogs (Funakoshi and Zotterman 1963)

In the present work the aim was that of studying the response of the pulpal receptors to the elevation of intrapulpal pressure The experiments were performed with 6 cats weighing from 2 5 to 4 7 kg They were anesthetized with i p chloralose The animals were ventilated by means of a Palmer ideal pump with a mixture of 25 % pure oxygen and 75 % of room air and a frequency of 30—34/min Both of the parameters were changed in accordance with the values of Astrup-analyses so that the experiments were performed under normal conditions of acid base balance The inferior alveolar nerve of the left side was made free by removal of the layer of bone from the lower margin of the mandibula by means of a drill and an osteostome A pool for nerve preparation was formed by the bone cavity and surrounding tissues and filled with mineral oil The nerve impulses were recorded monopolarly from the distal end of the cut nerve of the canine tooth by the use of platinum iridium electrodes. The signals were led to a Tektronix 122 preamplifier and on to a Tektronix 565 oscilloscope

A cavity was drilled in the canine tooth through the enamel and dentine to the pulpal surface Penetration was considered to be of adequate depth when clear pulpal tissue fluid exuded from the cavity A cannula made of an injection needle 0 8 mm in external diameter was fixed to the drilled cavity The pressure was measured from an outlet of a T shaped tube one end of which was fixed firmly to

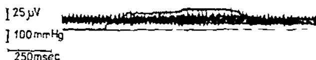


Fig 1 Influence of intrapulpal pressure elevation on afferent pulpal nerve fibre activity The figure illustrates that a rise in pressure induces an increase in afferentation The upper solid line represents the pressure curve and the lower the O level of pressure

a support and to the table with a view to preventing activation of the periodontal receptors of the canine tooth as a movement artefact during the course of pressure changes The polyethylene tube from the T shaped cannula was led to a Sanborn 267 bc pressure transducer on to a Sanborn 350—1100 c carrier preamplifier and then to the oscilloscope The signals were filmed from the screen with a Milano-Ardume oscilloscope camera at a film speed of 15 5 cm/s The pressure impulse ($I = \int p \times dt$) was calculated by application of the method of calculation to be found in Bergstrom (1966) Viljanen (1967) and Hakumaki (1970)

In all 6 animals an elevation of the intrapulpal pressure induced an increase in afferent pulpal nerve impulses of the canine tooth (Fig 1) An almost linear correlation was observable between the intrapulpal pressure impulse and the number of afferent pulpal nerve impulses during the time in question (Fig 2) A further observation was that a negative pressure also increased the nervous activity

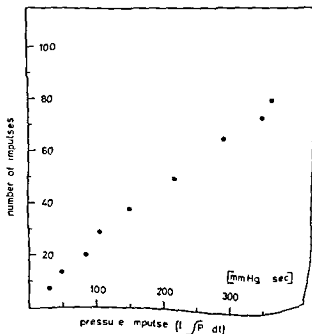


Fig 2 The number of pulpal afferent nerve impulses as a function of the pulpal pressure impulse during the course of one pressure elevation The figure indicates the existence of an almost linear correlation between these two parameters

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Acta physiol scand 1973 88 587—589

From the Brain Research Laboratory E blocket University Hospital Lund Sweden

Blood Flow and Metabolic Rate for Oxygen in the Cerebral Cortex of the Rat

By

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When studying the metabolism of the brain it is often an advantage to use small animals like rats However such studies have been hampered by the lack of suitable methods to measure the cerebral blood flow (CBF) and the cerebral metabolic rate for oxygen (CMR_{O_2}) The CBF methods hitherto described are either based on the tacit assumption that the indicator used is not diffusion limited (Kety et al 1969) or require a simultaneous determination of cardiac output (Goldman and Sapirstein 1973 Pannier and Leusen 1973)

We have applied the Kety and Schmidt (1945) method to measurements of blood flow in the rat brain utilizing inhalation of ^{133}Xe and blood sampling from the femoral artery and from the superior sagittal sinus Since the results demonstrate that the CMR_{O_2} obtained is considerably higher than in the human brain a preliminary account of the experiments is given

The experiments were performed on 300–470 g rats which were maintained on artificial respiration with 70 % N_2O and 30 % O_2 The body temperature was adjusted to 37 C and the Pa_{CO_2} to 35–40 mm Hg In two other groups the Pa_{CO_2} was either lowered to 10–15 mm Hg or increased to about 80 mm Hg A femoral artery was cannulated and the superior sagittal sinus was exposed for sampling of arterial and cerebral venous blood respectively In order to avoid a fall in blood pressure due to the repeated sampling fresh blood from a donor rat was infused during the sampling period

The CBF was determined with ^{133}Xe which was delivered from a rubber bag attached to the respirator After a saturation period of 20 min when the brain tissue has virtually reached equilibrium with arterial and venous blood the bag was disconnected and the CBF was calculated from the desaturation curves obtained by scintillation counting of the ^{133}Xe activity in the 10 [illegible] samples Each sample taken had a volume of about 50 µl and of this 25 µl was transferred with a Hamilton syringe to the bottom of a water filled test tube that was counted in a well type scintillation counter The oxygen content in arterial and venous blood was determined at the start and at the end of the desaturation period using polarographic measurements of P_{O_2} after liberation of oxygen with ferricyanide The CMR_{O_2} was calculated by multiplying the CBF with the arteriovenous difference in oxygen content

Fig 1 shows representative desaturation curves for hypocapnia normocapnia and hypercapnia respectively with the calculated CBF values The table gives the mean CBF and CMR_{O_2} values for the 3 levels of Pa_{CO_2} The reduction in Pa_{CO_2} to 13 mm Hg decreased CBF to 60 % of normal while Pa_{CO_2} 80 mm Hg increased CBF to

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Acetylcholine and Related Enzymes in Normal and Ligated Cholinergic Nerves from Torpedo marmorata

By

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Recent studies indicate a rapid proximo distal axonal transport of acetylcholine (ACh) in mammalian peripheral nerves (see *e g* Sastry 1956 Evans and Saunders 1967, Haggendal *et al* 1971 1973, Dahlstrom *et al* 1973 Saunders *et al* 1973) A redistribution of ACh and ChAc seems to occur at various times after axotomy and drugs which interfere with microtubules *i e* vinblastin and colchicine decrease ACh accumulation above a crush or a ligature (Dahlstrom *et al* 1973) The amount of ACh transported is small compared to that present at the synapses and its function is not yet understood

It is not yet clear if the transported ACh occurs freely in the axoplasm or if it is particulate Work on the subcellular distribution of ACh and enzymes in cat n ischiadicus suggests a transport compartment In such nerves various organelles and structures accumulate above a ligature and particles containing ACh seem to exist (Heilbronn *et al* 1973) However, the ACh content of the various subcellular fractions is very low which makes it difficult to interpret the results with certainty Therefore it was decided to see if the purely cholinergic nerves that lead from the electric lobes in the brain of *Torpedo marmorata* to its electric organs could be used for studies on the origin of synaptic ACh

The fishes were anesthetized in artificial seawater containing about 0 1 % of urethane To ligate nerves a cut was made from one of the blow holes towards the tail on the dorsal side of the animal A stretch of the relevant nerves was exposed between the skull and the gill arches and ligated with a fine thread Great care was taken not to sever any larger blood vessels After ligation the wound was closed and the fishes left in artificial sea water for 48—55 h The nerves of the opposite electric organ were used as controls At the end of the experiments the fishes were reanestethized ligated nerves were excised and 0 6—1 cm of the proximal and distal parts closest to the ligature were taken for analysis of ACh choline acetyltransferase (ChAc) acetylcholine esterase (AChE) thiamine pyrophosphatase (TPP) and in fractionation experiments some subcellular marker enzymes The enzyme methods are described elsewhere (Heilbronn *et al* 1973) Total ACh was determined after perchloric acid extraction of nerves frozen in liquid nitrogen In some experiments nerves were homogenized with a glass—glass Potter—Elvehjem homogeniser (2×10 strokes) in 0 32 M sucrose containing 0 05 M NaCl 5 mM Tris pH 7 4 and left for 20 min at room temperature to allow AChE present in the axons to destroy nonoccluded ACh The remaining ACh was then extracted as described above Sometimes 5×10⁻ M eserine was added to preserve free ACh Such samples were acid boiled for determination of total Ach A pellet spun down at 4500 rpm for 10 min from these preparations prior to gradient centrifugation contained only 4 % of the total amount of lactic dehydrogenase in the pellet ACh was mostly determined by the leech muscle assay occasionally by massfragmentography In some of the latter experiments choline was also determined Calculations were done per unit length or per g wet weight

TABLE I Activities of choline acetyltransferase (ChAc) and the marker enzymes acid phosphatase and thiamine pyrophosphatase (TPP) found in ligated (48 h) and control nerves

nr and part of nerve	Acid phosphatase nmol g⁻ h⁻	TPP nmol g hr^{-1}	ChAc cpm g⁻ min^{-1}
Proximal 1	14 [illegible]86	2916	44 643
Distal 1	10 309	3[illegible]7	(9 8)
Control 1	7 [illegible]8[illegible]	3431	—
Control 1	7 329	4300	12 3[illegible]8
Proximal 3+4	10 000	[illegible]867	19 600
Distal 3+4	9 173	3291	6 745
Control 3+4	8 194	388[illegible]	14 931

Homogenates of the nerve pieces showed that acid phosphatase a marker enzyme for lysosomes always was higher in ligated than in control nerves and higher in the proximal than in the distal part of the stump (Table I) TPP was in general very low and on the basis of the occurrence of this enzyme nothing can therefore be said about the origin of the many vesicles found in electron micrographs of ligated nerves ChAc is low in the normal axons increases proximally to the ligature and decreases distally (Table I)

An experiment with 8 pooled nerve axons ligated for 51 h and obtained from 2 fishes showed after homogenisation of the excised axon stumps in eserinized buffered sucrose clearly accumulation of free ACh proximal to the ligatures Distally in the cm nearest to the ligature the obtained values agree well with those of the unligated nerve Boiling of the samples at pH 4 [illegible] to release bound ACh results in considerably higher values for total ACh (Table II) This is probably not due to artificial vesicle formation upon homogenisation as only the supernatant and its nearest fraction contain lactic dehydrogenase activity when the homogenate is submitted to sub cellular fractionation Experiments with single nerve axons revealed differences in the total ACh content of the various cholinergic nerves either the values were measured with the leech muscle assay (3—14 nmoles per g) or by massfragmentography (1—8 nmoles per g) The concentration of choline in these nerves is about 10—30 times higher than that of ACh between [illegible]0—145 nmoles per g

TABLE II Fr[illegible] and total (acid bo[illegible]led sampl[illegible] ACh ± S D [illegible] buff[illegible]red sucrose homogenates obta[illegible]ned [illegible] h ligation f[illegible]om po[illegible]led p[illegible]oximal and distal [illegible]tumps of nerves f[illegible]om [illegible] *T[illegible] p[illegible] d[illegible] marm[illegible] ata* Oppos[illegible]te ner[illegible]s used a[illegible] [illegible]ontr[illegible]ls Number of e[illegible]timat[illegible]ons [illegible]n brackets

Nerve p[illegible]t	ACh		
	nmol cm fr[illegible]e	nmol g	total
Cont[illegible]ol	2800	6 7 ± 1 0 (3)	9 8 ± 3 0 (4)
Proxi[illegible]nal	5900	14 1 ± 1 1 (4)	42 5 ± 5 5 (3)
Distal	2860	7 0 ± 0 9 (3)	—

The present results show accumulation of ACh ChAc and acid phosphatase above a ligature in cholinergic axons of *Torpedo marmorata* and suggest a proximo-distal transport of ACh and these enzymes After homogenisation of axons part of the ACh present is found to be nonaccessible to AChE, particularly in the proximal stump of a ligated axon The presence of large amounts of choline and of the rather small but obvious ChAc activity demands a thorough analysis of the role of axonal ACh biosynthesis in the observed ACh increase above a ligature

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